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CONTENTS

The Relation of Blood Factors to Icterus in Foals 237
D. W. Bruner, E. F. Hull, and E. R. Doll

Transmission and Immunization Studies on Equine Influenza 243
T. C. Jones, Chester A. Gleiser, Fred D. Maurer, Maurice W. Hale, and Thomas O. Roby

Water-in-Oil Emulsions for Delaying Absorption of Penicillin in Horses 254
E. R. Doll, and M. Elizabeth Wallace

Penicillin Therapy in Chronic Bovine Mastitis. II. Penicillin Levels in the Udder During Treatment 259
R. A. Packer

Penicillin Therapy in Chronic Bovine Mastitis. III. Treatment of Mastitis 264
R. A. Packer

DDT and DDT Combined with Benzene Hexachloride for the Control and Eradication of Boöphilus Annulatus 270
N. G. Cobbett

The Potentiating Action of Sulfonamides on the Brucella Antibody-Complement System 277
I. Forest Huddleson

Motility of Bovine Spermatozoa 286
C. K. Rao, and G. H. Hart

Hemolymph Nodes in Small Ruminants 291
Zihni Erencin

The Control of Ovine Enterotoxemia by the Use of Clostridium Perfringens Type D. Bacterin 296
Earl M. Baldwin, Jr., L. D. Frederick, and J. D. Ray

A Technique for the Isolation of Newcastle Disease Virus, Using Streptomycin as a Bacterial Inhibitor 303
C. H. Thompson, Jr., and O. L. Osteen

Relative Activity of Sulfamethazine and Sulfaquinoxaline Against Eimeria Tenella Infection in Young Chickens 306
L. C. Grumbles, and J. P. Delaplane

Sulfamerazine in the Treatment of Fowl Cholera in Turkeys 310
J. O. Alberts, and Robert Graham

The Isolation and Identification of Fowl Plague Virus 314
H. E. Moses, C. A. Brandly, E. Elizabeth Jones, and E. L. Jungherr

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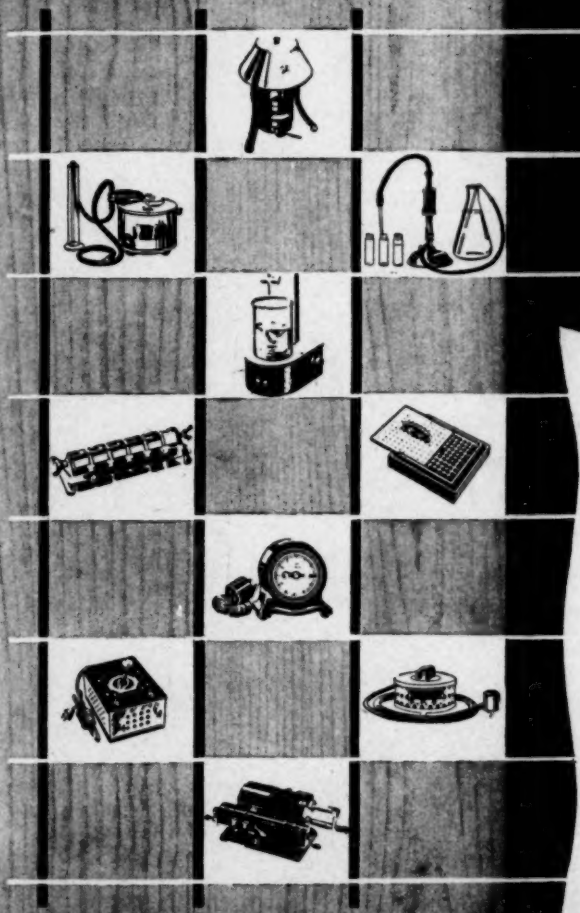

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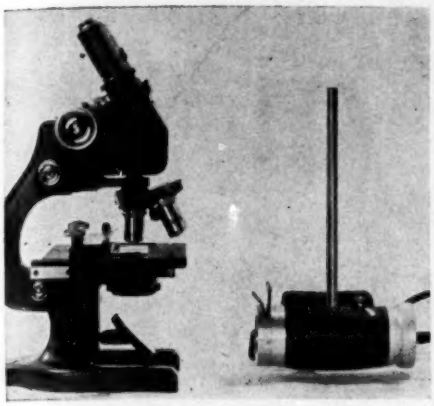

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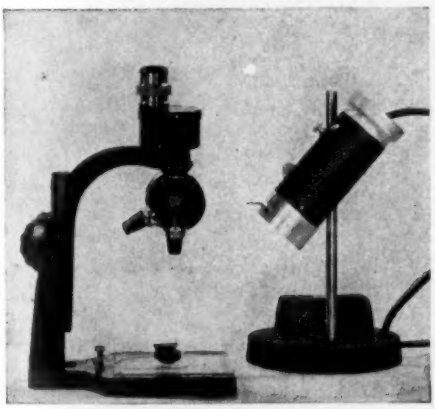

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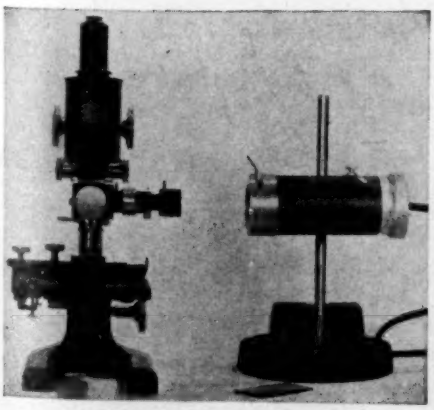

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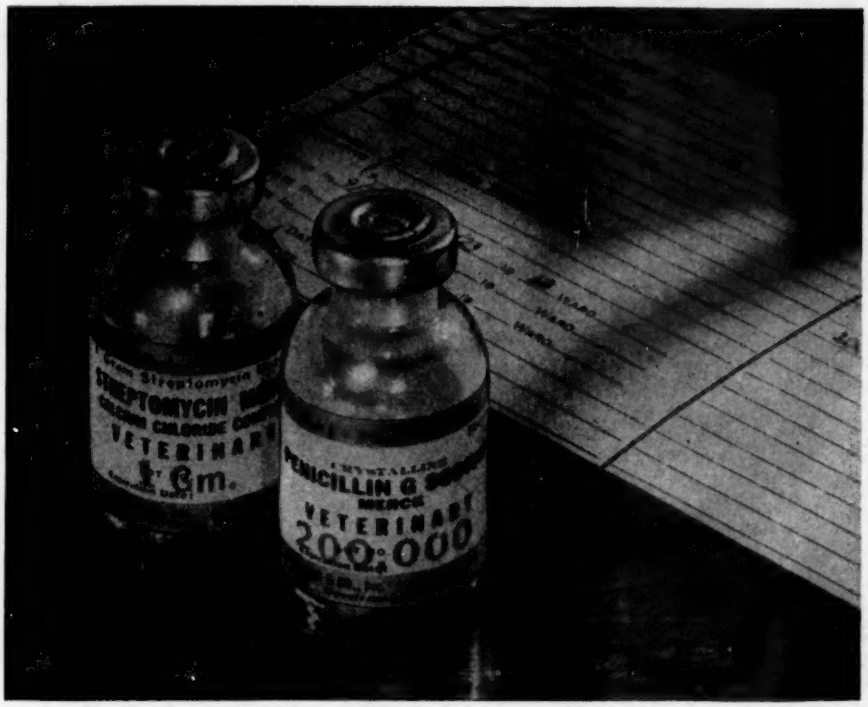

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VOL. IX JULY, 1948 No. 32

The Relation of Blood Factors to Icterus in Foals

D. W. BRUNER, D.V.M., Ph.D., E. F. HULL, D.V.M., and E. R. DOLL, D.V.M.

Lexington, Kentucky

BRUNER, Hull, Edwards, and Doll[1] described a condition of icterus in newborn foals and showed that 6 mares which had produced such offspring were sensitized to the erythrocytes of the stallions to which they were bred. One of the main characteristics of this type of icterus was its appearance in foals within twelve to ninety-six hours after they began to nurse. This indicated that the milk of the mare contained antibodies which were absorbed by the suckling foal. These antibodies attacked the erythrocytes of the foal and symptoms of icterus developed. That the natural transfer of passive immunity from the mare to the foal actually occurs through the colostrum was discussed by Bruner, Edwards, and Doll.[2] The more recent investigations by members of this department concerning hemolytic icteric foals are given in this paper.*

PROCEDURE

During the fall of 1947, it was established that 12 mares in the Bluegrass Area of Kentucky had produced icteric foals. These mares furnished material throughout their recent pregnancies and foaling periods. Their foals and the stallions to which they were bred also were considered. The 12 mares had been in stud for periods of six to eighteen years. One mare had produced 10 living foals, and each mare had produced at least 2 normal foals before giving birth to one which developed icterus. Prior to 1948, these mares had given rise to 21 icteric foals, of which 6 survived. The 21 foals were sired by 14 stallions, and 3 of the mares had icteric foals by more than one stallion.

Besides the 12 mares mentioned above, 9 produced hemolytic icteric foals for the first time in 1948. Eight of these mares had been in stud for a number of years and had produced living foals, but the first foal of the ninth mare died of hemolytic icterus.

Five, pregnant, experimental mares were immunized against the erythrocytes of the stallion to which they were bred. This was accomplished by giving 3 intravenous injections of 30 cc. of citrated stallion blood at seven-day intervals, followed by several weekly subcutaneous injections of 20 cc. each to maintain the serum titer at the desired level until the mare foaled. These injections were started about three months before the termination of pregnancy.

In making agglutination tests with various blood specimens, an initial dilution of 1:2 of serum was employed because the presence of small amounts of iso-agglutinins in the blood serums of many horses frequently will cause agglutination in undiluted serum. By starting with a dilution of 1:2, minor reactions can be ruled out. A 50 per cent washed cell suspension was used because it was determined that horse erythrocytes, unlike human cells, give more rapid and clear cut reactions in heavier suspensions. Tests also were made for the presence of hemolysins in the serums of mares producing icteric foals. This was done by placing measured amounts of washed erythrocytes directly into dilutions of fresh mare serum and by mixing mare serum, guinea pig complement, and washed erythrocytes. Antiserums were prepared by injecting rabbits with washed erythrocytes of the sires of jaundiced foals. The erythrocytes of a jackass also were employed.

The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station, Lexington, and is published by permission of the director.

Dr. Bruner is bacteriologist; Dr. Hull, professor of veterinary science and head of the department, and Dr. Doll, veterinarian in the Department of Animal Pathology, University of Kentucky, Lexington.

*Since this article was submitted for publication, the attention of the authors was drawn to the studies of Bessis and Caroli on the involvement of blood factors in icterus of newborn mules. Reports of their findings appeared in the Comptes rendus des séances de la Société de biologie., *141*, (1947): 387 and in the Comptes rendus des séances de l'Académie des Sciences., *224*, (1947): 969-971.

RESULTS

Of the 12 mares which had records of producing icteric foals, one was not bred in 1947 and another was found to be barren. The remaining mares gave birth to 10 apparently normal foals during the spring of 1948. Nine of these foals are alive and healthy at this time. One died of *Shigella equuli** infection. In investigating the relationship of blood factors in this disease, specimens were taken for the first time in the fifth to seventh month of pregnancy. In each case, the serum of the mare was matched with the erythrocytes of the stallion to which it was bred. These tests showed that the serum of only one of the 10 mares did not agglutinate the erythrocytes of its mate in a dilution of 1:2. That mare had produced a foal in 1947 which died of hemolytic icterus. The mare then was bred to a different stallion whose cells were compatible with its cells, and the mare gave birth to a normal foal in 1948. During pregnancy, the mare's serum titer against the erythrocytes of the stallion that sired its 1947 foal gradually dropped from 1:32 in October, 1947, to 1:8 at the time it foaled. The foal did not develop icterus even though its serum after nursing showed a titer of 1:8 for the erythrocytes of the sire of the icteric foal of 1947.

The other 9 mares which were bred to stallions having incompatible erythrocytes showed varying degrees of agglutination reactions when their blood samples were cross matched. In no instance did the serums of the stallions agglutinate the erythrocytes of the mares. Periodic cross matchings as the pregnancies drew to term showed that the titers of certain mares increased; others remained unchanged; and some dropped. In all cases where the titer of the mare increased during pregnancy, a nurse mare was recommended for the foal. As soon as the foal was born, tests were made with its blood, the sire's blood, and the blood and milk of the dam. The foal was not allowed to nurse its dam until the outcome of the tests was known. If the erythrocytes of the foal were not compatible with the serum of the mare and agglutinated to a titer of 1:4 or more and to a titer of 1:16 or higher with the mare's colostrum, it was dangerous for the foal to nurse its dam. In this connection, it was shown that the stallion's red blood cells also should be used to determine whether the foal should nurse its dam, because the erythrocytes of the newborn foal did not always lend themselves readily to agglutination, especially in those instances where the mare's serum showed marked advances in hemagglutinin titer during the period of pregnancy.

Of the 10 foals born, nurse mares immediately were used for 4. One of these foals died of *Shig. equuli* infection within two days after birth. The others have done very nicely on their foster mothers. Only 1 of them was given a blood transfusion from a mare whose erythrocytes were compatible with the serum of the foal, as a precautionary measure against infection before placing it on the nurse mare. The fifth foal was permitted to nurse its dam even though tests showed that procedure to be dangerous. It developed a severe case of icterus but recovered following a blood transfusion and a switch to a nurse mare.

Four foals were allowed to nurse their dams after the necessary tests were made. In one case, the mare had been bred to a stallion whose cells were incompatible with those of the mare, but the foal had inherited cells like its dam and not like its sire. In this instance, the titer of the mare did not increase during pregnancy and, since the foal's red blood cells were compatible with those of the dam, it was allowed to nurse at will. This foal never showed any symptoms of hemolytic icterus. In the second case, the foal was permitted to nurse because the mare had a titer of only 1:2 in its serum and of 1:32 in its milk. This foal developed icterus but recovered without treatment. The third foal showed such weak reactions with its dam's serum and

*Van Straaten actually applied the binomial *Shigella equuli* to this microörganism (van Straaten, verslag van de Werksaamheden der Rijksserumirichting, Rotterdam, (1918): 75). Unfortunately, de Blieck and van Heelsbergen (Lydschr v. Diergeneesk, *46*, (1919): 492) in quoting from van Straaten used the *Shigella equirulis*, and this designation has appeared in recent literature (Dimock, Edward, and Bruner: Kentucky Agric. Expt. Sta. Bull. 509, (1947): 16).

colostrum that a nurse mare was not used. It did not develop jaundice. The fourth foal's erythrocytes reacted at 1:2 with the dam's serum and 1:32 with the dam's colostrum. It was decided to milk out the colostrum by hand stripping the mare at hourly intervals for about eight hours. At the end of that period, the udder secretion had the appearance of normal milk and a titer of 1:8 for the foal's red cells. The foal was allowed to nurse and, although it developed a slight amount of icterus within eight hours after starting to nurse, no further treatment was needed.

Tests made with these 10 pregnant mares just before parturition showed serum titers varying from 1:2 to 1:64 and colostrum titers of 1:8 to 1:128 against the erythrocytes of the sire. Within twelve hours after parturition, their serum titers had not changed, but the titers of the milk of those being nursed ranged from less than 1:2 to 1:8. Within a week, all of these mares had titers of less than 1:2 in their milk. The titer of a mare's colostrum usually is higher than that of its serum. If the foal is active in nursing, the udder secretion of colostrum is changed to milk in less than twelve hours and, at the same time, its concentration of antibodies is reduced. That small amounts of antibodies continue to be secreted in the milk was demonstrated by Bruner, Edwards, and Doll.[2] Mares continued to hold their serum titer levels for several weeks after parturition. Compatibility tests made at four to five months after birth indicated that the antigens of the erythrocytes of the foal remained constant.

The series of agglutination tests on the 10 pregnant mares and their newborn foals established some general rules to follow in adopting the proper course for the care of the foal. If the serum titer of the mare is no higher than 1:2 and that of its colostrum no higher than 1:8 when tested against the erythrocytes of both the newborn foal and the sire, it appears that the foal safely may nurse its dam. If the serum titer of the mare is 1:4 and its colostrum titer is 1:16 or higher, the foal likely will develop icterus and may or may not recover without treatment. When the serum titer of the mare is higher than 1:4 and its colostrum titer is higher than 1:16, it is dangerous for the foal to nurse its dam.

While this work was in progress, 9 additional mares not previously known to be icteric foal producers presented their first cases in 1948. Three of the 9 foals died and were brought to the laboratory for postmortem examination before the diagnosis was made. One of these foals died within twelve hours after birth. The serum of its dam showed a hemagglutinin titer of 1:64 when tested with the erythrocytes of the sire. This is one of the highest titers we have seen in a field case. The other 2 lived twenty-eight hours and seven days, respectively. Blood from mares of both foals agglutinated the red blood cells of the respective sires.

In the 6 remaining foals, hemolytic icterus was diagnosed within twenty-four to forty-eight hours after birth. Their red blood cell counts ranged from 2,800,000 to 3,200,000 per cmm. of blood. Three of these sick foals were treated by blood transfusions of compatible blood and continued to nurse their dams. Only one recovered. Three were given transfusions of compatible blood and placed on nurse mares. Two of these foals recovered. In all cases, the serums of the mares strongly agglutinated the cells of their foals. Since all 6 foals had nursed, the milk of the mares appeared to be free of agglutinins when viewed by agglutination tests. That the tests were not sensitive enough to detect the small amount of antibodies secreted in the milk was indicated by previous results.[2] As knowledge was gained in the treatment of hemolytic icterus cases, it became evident that it was necessary to remove at least 1,500 cc. of blood from a sick foal and replace it with at least 2,000 cc. of compatible blood. It also was necessary to place the foal on a nurse mare which was not secreting hemagglutinins specific for the foal's red cells.

In the field cases of hemolytic icterus studied during the past foaling season, it readily was demonstrated that the serum of the sensitized mare not only carried hemagglutinins against certain types of erythrocytes but also possessed hemolysins

for those red cells. When the proper type of erythrocyte was exposed to the action of fresh serum from a mare consistently producing icteric foals, complete hemolysis occurred in dilutions up to 1:10. It also appeared that the hemolysin titer regulated the rapidity of the onset of symptoms of icterus.

EXPERIMENTAL MARES

Three of the 5 pregnant, experimental mares used in this study were maiden mares, and the others had produced normal foals in past seasons. At the start of the experiment, these mares did not carry iso-agglutinins in their serums at a dilution of 1:2 for the erythrocytes of each other or for the stallion to which they were bred. After 3 intravenous injections, it was decided that the 3 maiden mares would not develop antibodies against the stallion's red blood cells. In expanded cross matching tests with antiserums from 20 sensitized mares, it was concluded that the cells of the 3 refractory mares and the stallion were quite similar if not belonging to identical blood groups.

The two remaining mares developed agglutinins against the injected erythrocytes of the stallion. The first of these 2 mares to foal showed a serum titer of 1 : 2 and a colostrum titer of 1 : 16 when tested against the red blood cells of its newborn foal. The foal did not develop icterus. The second mare received three intravenous and two subcutaneous injections of the stallion's erythrocytes. The last injection was administered four weeks before parturition and the mare's hemagglutinin titer continued to rise steadily throughout this period. The mare showed a serum titer of 1 : 512 and a colostrum titer of 1 : 1,024 against the cells of its newborn foal. The erythrocyte count of the foal was 12,800,000 per mm.^{3} and the cell volume was 43. Its blood contaned no detectable hemagglutinins. After the foal was with the mare for ten hours, nursing at will, the red cells of the foal were agglutinated strongly in its own serum. The erythrocyte count was approximately 5,600,000 per mm.^{3} and the cell volume 18. The hemagglutinin titer of the colostrum had dropped from 1 : 1,024 to 1 : 32. At this time, the foal showed little evidence of icterus, but at twelve hours a definite yellow color had appeared in the scleras. Within twenty-four hours, the red cell count on the foal dropped to 2,800,000, the cell volume to 14, and the icteric appearance of its eyeballs and mucous membranes was strongly evident. The hemagglutinin titer of the mare's milk was 1 : 16. On the third day, the red cell count of the foal had climbed to 3,800,000 even though its red blood cells continued to be agglutinated in its own serum. The titer of the milk was 1 : 8. The foal's serum was quite yellowish in color and its urine was yellow. By the end of one week, the foal was recovering from the attack of icterus. Its blood count was 5,600,000, cell volume 20, and its scleras were only slightly yellow. Its red blood cells were no longer agglutinated in its own serum, and the milk titer of the mare was less than 1 : 2. The hemagglutinin titer of the mare's serum maintained the level of 1 : 512. As mentioned above, the mare's titer had continued to rise during the last four weeks of pregnancy even though it received no injections of the stallion's blood. After parturition, the mare's titer no longer increased.

Two 15-day-old foals were given 500 cc. each of plasma on successive days from a mare which had produced icteric foals and showed a serum titer of 1 : 32 when tested with the erythrocytes of the foals. The serum of this mare also hemolyzed the red cells of the foals *in vitro* in a dilution of 1 : 10. Both foals developed icterus to a marked degree. Their blood cell counts dropped from 9,000,000 per mm.^{3} to less than 2,100,000. Both recovered from the hemolytic icterus without treatment. An attempt to produce icterus by placing plasma from the same mare in the stomach of a 5-day-old foal was not successful even though the cells of the foal were of the proper type. It was learned later that a 5-day-old foal may lack the ability readily to absorb immune bodies placed in its digestive tract.

DISCUSSION

From the preceding experiments, it appears that blood factors play an important rôle in the production of hemolytic icterus in the newborn foal. Bruner, Hull, Edwards, and Doll[1] stated that this condition is not caused by the Rh factor of man. In horses, it appears that the mare becomes sensitized to a certain type of erythrocytes and from then on produces an icteric foal whenever it is bred to a stallion that transmits that type of erythrocytes. The manner by which the mare takes on this particular characteristic is not known. It is possible that the sensitization takes place through the breakdown and absorption of parts of the fetal placenta. Placentas of newborn foals frequently have necrotic areas or show other evidence of inflammatory changes and destruction of tissue. It is known that once a mare has become sensitized it may produce an icteric foal when bred to any stallion whose erythrocytes are incompatible to the mare.

Furthermore, the mare frequently will not agglutinate the erythrocytes of all the stallions that have sired icteric foals.

It appears that hemolytic icterus in the newborn foal is connected with individual blood groups rather than an Rh-like factor in a very high percentage of individual horses. The 32 hemolytic icteric cases listed were sired by 22 stallions. Of these stallions, the erythrocytes of only 1 were incompatible with all of the 21 mares that have produced icteric foals. An antiserum prepared by injecting the washed erythrocytes of this stallion into a rabbit could not be used to select individuals producing icteric foals. Absorption of this antiserum by the erythrocytes of certain mares producing icteric foals would exhaust it completely of hemagglutinins. The use of jackass red cells to develop a serum comparable to the rhesus monkey antiserum used in human investigations was of no avail. Although the anti-jackass-erythrocyte serum agglutinated horse erythroyctes readily, mares which had produced icteric foals were able to exhaust it of all heterologous agglutinins.

Since mares naturally transfer passive immunity through their colostrum to the newborn foals, the reason for the appearance of icterus in an apparently normal and active foal within twelve to ninety-six hours after its birth readily is explained. The fact that the concentration of antibodies is always high in the colostrum and drops to a level too low to be detected by the ordinary agglutination test after twelve hours of nursing does not mean that the foal no longer receives any antibodies from the milk. A foal whose red cell count has dropped below 3,000,000 per mm.^{3} should be taken away from its dam since any further loss of erythrocytes through the taking up of even small amounts of antibodies from the mare may cause death. In dealing with bacterial antigens which can be used to stimulate high antibody titers, it was demonstrated that these substances are secreted in small amounts in the mare's milk for several weeks after foaling.[2] It is not unreasonable to assume that the same thing holds true in the transfer of hemagglutinins.

In this study, it also was shown that the same stallion bred to 2 mares capable of producing icteric foals sired both a normal and an icteric foal. Tests made with the erythrocytes of the foals showed that the stallion did not transmit its type of blood cells to the normal foal but did transmit them to the foal which became icteric.

It is not entirely clear why the artificially produced case of icterus in a newborn foal did not terminate in a fatality, since its dam developed a higher serum titer and a higher colostrum titer than did the dams of fatal field cases. This mare did not develop a hemolysin titer effective in a dilution of 1:10, while hemolysins were present at that level in the dams which produced fatal cases of icterus. It is quite likely that the ability of the sire to stimulate the production of hemolysins along with hemagglutinins is a determining factor in the outcome of the disease in newborn foals. It is of interest that the titer of this mare continued to rise for four weeks after the last injection and then stopped its climb immediately on parturition.

Although it is apparent that some mares which have become sensitized to certain types of erythrocytes will produce icteric foals when bred to incompatible stallions, no adequate tests have been devised to date to select first offending mares. It would be possible to test all mares against the stallions to which they are bred and select the susceptible mares. These tests would have to be made for each mare near the termination of pregnancy and would involve considerable time and expense. Further study of the question, especially the establishment of the blood types in horses, might help solve the problem; at least all stallions could then be typed and matings selected to prevent the production of icteric foals without doing numerous cross matching tests for each individual mare.

Since Schneider and Szathmary[3] indicated that the pig falls in the same category as the horse as far as the transfer of passive immunity to the offspring is concerned, it is highly probable that certain types of anemia seen in baby pigs are caused by blood factors which operate in

a manner similar to those found in the mare.

SUMMARY

Cases of hemolytic icterus in newborn foals occurred when mares sensitized to certain types of erythrocytes were bred to stallions which transmitted that type of blood cells to their offspring. The condition was observed in apparently normal newborn foals after they had obtained from the dams' milk the antibodies which destroyed their erythrocytes. A sensitized mare which was bred to a stallion whose cells were compatible with the mare's cells did not produce an icteric foal. Susceptible foals sired by stallions whose cells were not compatible with those of the mare did not develop icterus if placed on nurse mares immediately after birth. Upon the appearance of hemolytic icterus, the foal was removed from its dam and 1,500 cc. of its antibody-laden blood was withdrawn and replaced by 2,000 cc. of compatible blood.

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Transmission and Immunization Studies on Equine Influenza

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The purpose of this report is to present data from certain experimental studies on equine influenza, which were started in 1939 in the Army Veterinary Research Laboratory at the Front Royal Quartermaster Depot (Remount), Front Royal, Va., and continued in the new location of the laboratory at Fort Robinson, Neb. The technical operation of this project was under the supervision of the Research and Development Board of the Surgeon General's Office, War Department, Washington, D. C.

The general objective of all work in this project was to develop means of prevention of morbidity and mortality due to equine influenza and its complications in Army animals. Isolation of the virus of equine influenza, its transmission by serial passage in colts, the search for suitable, small, laboratory animals, attempts to cultivate the virus, its neutralization with convalescent horse serum, use of the virus as a vaccine, and the nature of the experimental disease will be described in this report. It is hoped that the experiences outlined will be of value to other research workers in connection with this and similar problems.

MATERIALS AND METHODS

Sources of Virus.—Horse blood was collected aseptically by bleeding from the jugular vein and defibrinated by shaking with glass beads.

Nasal washings were collected in the following manner: 30 to 100 cc. of sterile nutrient broth was injected into the upper nasal passages of a horse through a 30-cc. Luer syringe attached to a stiff rubber catheter about 18 in. long. As the broth was forced up into the nostrils, the horse's head was held down and the return flow caught in a sterile basin.

From horses in the early stages of influenza, killed by shooting through the brain, nasal and turbinate mucosae were collected in the following manner: The head was skinned, placed with the mandible uppermost, and split adjacent and parallel to the nasal septum with a heavy meat cleaver. The halves of the head were then pulled apart, exposing the nasal cavities and turbinates. The nasal and turbinate mucosae were stripped off with sterile scissors and forceps and placed in 100-cc. lusteroid tubes. The tubes were sealed with cork stoppers, secured by adhesive tape, and immediately immersed in solid carbon dioxide (dry ice)-alcohol mixture until frozen.

Specimens of spleen were collected by opening the cadaver from its left side, cutting the ribs and diaphragm, and lifting them away to expose the viscera. Care was taken to use only sterile instruments in removing the spleen and cutting it into pieces about 1 in. square and placing it in 100-cc. lusteroid tubes. These specimens were also frozen in the dry ice-alcohol mixture. This simple technique obviated searing of the spleen and avoided bacterial contamination. Specimens of lung, lymph nodes, and liver were collected under the same conditions.

Inoculation of Animals.—Intranasal inoculation of horses was accomplished by the use of a 30-cc. Luer syringe attached to a human-type, rubber urethral catheter which had been stiffened by attaching a piece of wire along its length with adhesive tape. The supension of virus material was drawn up into the syringe through the catheter; then the catheter was introduced as far as possible into the nasal passages of the horse and the material injected under considerable pressure. Air was then drawn into the syringe and ejected several times to force the material remaining in the syringe and catheter into the nostril. The usual amount of material injected intranasally was 25 cc. A sterile towel was held over the catheter and nostrils to prevent gross contamination and loss of the virus suspension. Young calves also were injected intranasally by a similar technique.

Intranasal injections in mice, rats, ferrets, hamsters, rabbits, guinea pigs, and cats were accomplished under ether anesthesia. The virus suspension was usually placed drop by drop on the external nares using a Luer syringe or special glass pipettes calibrated to deliver 0.03-cc. or 0.05-cc. amounts, but in

From the Veterinary Corps, U. S. Army.

some experiments the virus suspension was introduced into a closed chamber in the form of a persistent mist. This was accomplished by placing the suspension in a glass nebulizer (DeVilbiss No. 40) which was attached to a glass fractionating column about 75 cm. high. Air was forced through the nebulizer, up the fractionating columns (where coarse particles were separated and returned by gravity to the nebulizer) into the closed chamber containing the experimental animals. The exhaust was carried through a copper coil where it was heated with a Bunsen burner to kill any residual virus. Animals were left in this infected atmosphere for varying periods of time. This method resulted in fatal infections from swine influenza virus in mice, after a ten-minute exposure.

Various procedures were employed to lower the resistance of experimental animals to the virus of equine influenza. Animals were chilled before and after inoculation, inoculated repeatedly with virus supensions, and in some instances the spleen was removed before exposure.

Preservation of Virus.—All virus-containing tissues, prior to 1942, were stored in 50 per cent glycerin solution, buffered at pH 7.0, and kept in the freezing compartment of an electric refrigerator at approximately 0 C. During 1942, and subsequently, such materials were sealed and placed in sterile lusteroid or pyrex tubes, frozen quickly by immersion in a dry ice-alcohol mixture and then kept at −70 C. in a dry ice cabinet. The low temperature thus attained proved to be essential for the adequate preservation of the equine influenza virus.

Preinfection Serum.—Blood was collected aseptically from horses preceding natural or experimental infection and allowed to clot. The clear serum was pipetted off and stored in 60-cc. rubber stoppered vials at 5 C. without preservation. Serum was not considered to be preinfection until the animal from which it was collected was proved susceptible to equine influenza upon exposure to the disease.

Acute Phase Serum.—The serum was collected in the same manner as preinfection serum, but during the period when the horse was showing early acute symptoms of equine influenza.

Convalescent Serum.—Serum from horses during convalescence, usually fourteen days or

TABLE I—Susceptibility of Experimental Animals to the Virus of Equine Influenza

No. exptl. trials	Species and No. of animals used	Primary source of virus (from horses)	Route	Serial passages (No.)	Results
15	Horses (64)	Nasal washings, defib. blood; lung, turbinate & nasal mucosae	IN SC IV IT	22	Virus successfully maintained.
149	Mice (2271)	Same as above	IN IC IP	19	Pneumonia frequent result of bacterial infection; virus not established in mice.
49	Ferrets (309)	Same as above	IT IN IV SC	37	Unable to maintain virus in ferrets. Pneumonia and death result of bacterial infection. An infection (possibly equine influenza), carried through ferrets, trans. to horses.
2	Rats (39)	Horse lung, nasal mucosae & nasal washings	IT	7	Trace lesions first passage; no infection established.
6	Hamsters (91)	Bronchial exudate, nasal mucosae defib. blood: spleen	IN	9	No symptoms or lesions produced.
2	Guinea pigs (28)	Horse lung and defib. blood	IC	13	No symptoms or lesions observed.
1	Swine (5)	Defib. blood	IN	5	No symptoms or lesions due to equine virus.
2	Cats (24)	Defib. blood & nasal washings	IN	10	Mild symptoms or lesions due to equine virus.
1	Cotton rats (37)	Lungs & nasal mucosae	IN	10	No symptoms or lesions.
1	Calves (2)	Nasal mucosae	IN	2.	Moderate fever; no virus established.
1	Rabbits (22)	Nasal mucosae; defib. blood & spleen	IN IV	10	Alternate passage between colts and rabbits. No infection in rabbits.

IN = intranasally; IV = intravenously; IT = intratracheally; SC = subcutaneously; IC = intracerebrally; IP = intraperitoneally.

more after onset of symptoms, was collected and stored aseptically as described.

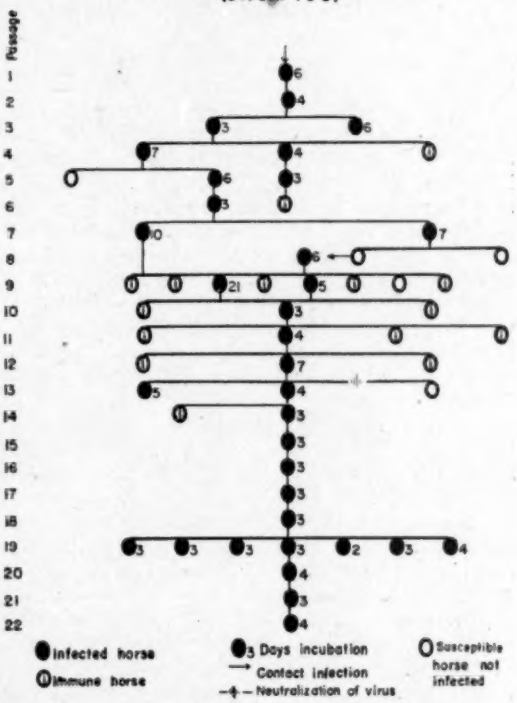
Preparation of Frozen Virus Vaccine.—From strain 183 virus, to be described later, living vaccine was prepared, using infected spleens of young colts as the source of virus. The spleen had to be harvested within the first few hours after the onset of the thermal reaction, in order to secure virus of infective titer. The spleen was removed aseptically and stored in the dry ice chest at –70 C. until needed in preparation of vaccine. The frozen spleen was later removed from the dry ice chest and pieces broken off with a sterile bone chisel and hammer. The pieces of spleen were weighed in a tared glass beaker and then transferred to a glass Waring blender cup which had been chilled in the refrigerator. A portion of the cold diluent was added, and the cup was placed in the refrigerator at 4 C. A physiologic solution of sodium chloride, buffered at pH 7.2 and containing 20 per cent preinfection horse serum was used as a suspending agent. Grinding was started as soon as thawing of the spleen became noticeable. The connective tissue was removed by pouring the resultant suspension through a cold porcelain Büchner funnel fitted with 16-mesh copper screen, all of which had been well rinsed with cold, buffered saline solution. The remainder of the diluent was added to make a 10 per cent suspension. The mixture was then centrifuged at 500 rpm. for two minutes at approximately 10 C., the supernatant poured off and bottled in 10-cc. quantities in 50-cc. rubber stoppered vials, shell-frozen, and held at –70 C. until used for animal inoculation or desiccation. All these operations were conducted under strict aseptic conditions, and cultures were made to determine bacteriologic sterility of the suspension before inoculation into animals. An aliquot of each lot of vaccine was also inoculated into a susceptible colt which must show a typical, febrile reaction with complete recovery, before the vaccine could be released. Careful observations were continued for at least thirty days to be certain that contamination with the virus of equine infectious anemia had not occurred. The shell-frozen suspension was thawed in cold water just prior to inoculation.

Preparation of Desiccated Vaccine.—The virus suspension described could be used only in close proximity to the laboratory as it required maintenance at –70 C. It was, therefore, desirable to prepare a more stable suspension. This was successfully accomplished by desiccation of the virus suspension, using the lyophile method. Suspensions of splenic tissue, freshly prepared, were removed from the dry ice chest and the 50-cc. bottles quickly attached to the manifold of the desiccating apparatus. The vials were attached one after another, allowing an interval to restore the vacuum after evacuation of air from the preceding vial. The pressure was maintained below 100 μ of mercury for the next twenty-four hours by an efficient high vacuum pump, the moisture being trapped in a condenser which was surrounded by dry ice.[1]

Each vial was removed from the manifold after desiccation for twenty-four hours and quickly stoppered aseptically under a vacuum of approximately 200 μ of mercury. The stopper was sealed by dipping into a 10 per cent acetone solution of isobutyl methacrylate and tested for vacuum with a spark coil tester. Analysis of samples of the final product revealed the residual moisture content to average 0.028 per cent by weight.

Table 2

Passage of Equine Influenza Virus through Horses (Strain 183)



—*Negative No. 102383 Army Institute of Pathology*

Selection and Isolation of Horses. — The most critical requirement in experimentation with equine influenza was the procurement of suitable horses for experimental purposes. It was essential that the animals be free from the disease or exposure thereto and still be fully susceptible. It was therefore necessary to know the complete veterinary history of each animal to be used for testing the virus. Young foals, 3 months to 1 year of age, were found to be most adaptable for transmission

of the virus, although both older and younger animals were occasionally used. The most suitable foals had been produced by immune dams and raised in an isolation area. They were weaned at least three weeks before exposure to equine influenza virus to avoid possible passive immunity which might have been gained from the mares' milk. A few foals were successfully raised from birth on a formula containing cow's milk, dextrose, brewer's yeast, lime water, and haliver oil, which obviated all possibility of such passive immunity. Accurate records of all illnesses were kept from birth assuring the susceptibility of each animal. Animals which had been in sales stables, stockyards, or railroad cars at any time were unacceptable for the experiments.

A concrete stable had been especially constructed for the research on infectious diseases, with stalls arranged to insure complete isolation. Each stall was approximately 12 ft. square, with a large, glazed window to permit observation from the central hallway and a cubicle about 5 ft. square in one corner, from which one door opened into the stall and one into the hallway. The cubicle contained a small wash basin equipped with soap and brushes, and a shallow depression in the floor in which a 5 per cent solution of liquor cresolis compositus was kept at all times. The depression was so constructed that it could be cleaned by brushing its contents into the stall. The attendant entered the stall through the cubicle, where he was required to put on boots and coveralls, scrub his hands and arms, and walk through the liquor cresolis suspension. Thus prepared, he cared for the horse and cleaned the stall. Manure and soiled straw were removed from the stall into a concrete box against its outer wall. A hinged lid on top of the box opened to admit the refuse which was later removed through a door on the outside of the stable. All brushes, brooms, manure forks, and thermometers were cleaned, sterilized, and along with feed, placed in the stall at the outset of the experiment and not removed until its termination.

When the attendant was through caring for the horse, he returned to the cubicle. He cleaned his boots thoroughly with a brush while standing in the liquor cresolis suspension, then removed and left them, along with the coveralls, in the cubicle. When he had removed these contaminated garments, he scrubbed his hands and arms before leaving the cubicle. The construction of the building and this program of disinfection and cleaning combined to prevent the occurrence of accidental cross infection.

ATTEMPTS TO INFECT LABORATORY ANIMALS WITH EQUINE INFLUENZA VIRUS

It was realized at the outset that study of the virus of equine influenza would be greatly facilitated if the virus could be established in small laboratory animals. Although considerable effort was expended, no suitable small animal was found in which the virus could be easily maintained and studied. Thus, it was necessary to use horses in all experiments in which virus identification was required, a handicap which greatly prolonged the time required, and increased the difficulties inherent, in the study of the equine influenza virus.

Several encouraging tests were completed in the winter of 1941-1942, in which an infection was encountered in ferrets following intranasal injection of material from infected horses. This ferret disease was an upper respiratory infection with febrile manifestations, lasting about one week, usually with complete recovery. It was transferred through six serial passages by intranasal inoculation of ferret nasal washings and by direct contact between ferrets. A horse injected with ferret materials (nasal washings, lungs, nasal epithelium) showed symptoms of equine influenza. Blood from this horse produced a febrile upper respiratory disease in a ferret. An infection of canine distemper appeared in the ferret colony at about this time, interrupting the experiments with ferrets until distemper could be eliminated. In the interim, the experimental ferret disease was lost and could never be recovered. It was, therefore, impossible to determine whether the ferret disease was due to equine influenza or to some intercurrent infection. However, it was proved by cross immunity tests that this ferret disease was different from the canine distemper infection which intervened so disastrously. In spite of all subsequent failures to establish this infection in ferrets, the possibility remains that some strain of equine influenza virus might be transmissible to ferrets, a possibility which justifies the recording of this experience.

The results of attempts to infect laboratory animals with equine influenza virus are outlined in table 1. These experiments were conducted over the course of several years, and minor changes in technique were made in many of them. Only the major differences are indicated in the table. Animals were inoculated with infective materials in many different ways: intranasally, subcutaneously, intravenously, intratracheally, intracerebrally, and intraperitoneally. It will also be seen that the virus was maintained by serial passage in colts, the details of which will be discussed in the section of the paper which follows. Aside from the inconclusive experience with ferrets, the virus was not infective for laboratory animals, even by serial passage. Large numbers of mice and ferrets and somewhat smaller numbers of hamsters, rats, guinea pigs, cotton rats, cats, swine, calves, and rabbits were used.

EXPERIMENTAL TRANSMISSION OF EQUINE INFLUENZA IN HORSES

The virus of equine influenza was transmitted in series from horse to horse and maintained in a viable state from 1941 to the present writing.

The history of strain 183 virus is outlined in table 2. This table illustrates the passage of the virus through 22 consecutive series in colts. It will be noted that several animals proved to be immune to the disease. Immunity due to previous natural exposure to the disease could be detected only by inoculation of living virus. Failure to establish infection was also experienced in some instances in which the colt later proved susceptible. These failures were reduced to a minimum as more was learned about the virus and techniques were improved. The first horse in the series was exposed to the disease by being placed in a paddock with several horses with clinically active equine influenza; six days later it contracted the disease. The animal was then killed and tissues collected for transmission to the

TABLE 3—Attempts to Culture Equine Influenza Virus in Chicken Embryos

Exper. No.	Route	Inoculum	No. of pass.	Length of incubation (hours)	Materials harvested	Age of embryos (days)
1	½ ys ½ iv.	blood	21	48	embryos	9-10
2	½ ys ½ iv.	filtered tracheal exudate	35	48 72	embryos	10
3	½ ys ½ iv.	filtered nasal washing	30	48	embryos	10
4	½ ys ½ iv.	lung	22	48	embryos	10
5	amn. & ca. after 8th pass.	blood	117	72	memb. & organs	12
6	ca.	blood	36	72	organs fl. & memb.	9-12
7	ca. & all.	equal parts leucocytes killed streptoccci	40	24, 48 72	fl. (all. & amn.)	9-12
8	ca.* all. cavity	lung & turbinates	5	72	embryos	8-12
9	ca. & all. space	turbinates	35	48	all. fl.	10
10	embryo	turbinates	66	24, 48 72	all. fl. & tracheas	13
11	ys	spleen	18	48	all. fl. & ys	9
12	amn.	spleen	10	96	mem., lungs, tracheas, other organs showing lesions	11
13	amn.	spleen	4	72	amnions & amn. & all. fl.	10
14	ca.	spleen	13	72	memb. & fl.	12
15	ca.	spleen	15	48	memb. & fl. lungs, spleen	12
16	ca.	spleen	7	72	memb. & fl.	12
17	iv.	spleen	5	48	blood	13

ys = yolk sac; iv. = intravenous; ca. = chorioallantoic; Pass. = passage; memb. = membranes; all = allantoic; amn. = amnionic; fl. = fluids.

Exper. 1, 2, 3, and 4—Embryos ground in broth, centrifuged, supernatant used as inoculum.

Exper. 5—Half the eggs kept at 35C. after 25th passage. Chilled at room temperature twenty-four hours after thirtieth passage. Bits of horse splenic tissue, testicle, and ovarian tissue were deposited on ca. m. Streptococci added once.

Exper. 6—Amnionic tissue from newborn foals placed on ca. m. Inoculated with virus in forty-eight hours.

Exper. 7—Streptococcus culture from acute influenza case. Leucocytes from blood drawn at peak of initial temperature rise.

Exper. 8—Use of 1:10,000 zephiran to remove contaminating bacteria in original inoculum.

Exper. 9—Zephiran and ultracentrifugation used to remove contaminants. 10^1 and 10^2 dilutions of virus used. Cysteine used to prevent oxidation of virus.

Exper. 10—Tracheas suspended in allantoic fluids. Hirst technique of inoculation used.

Exper. 11—Splenic suspension 10% used in dilution of 10^1 and 10^2. Harvested materials also diluted 10^1 and 10^2.

Exper. 12—Amnionic route through air cell; 10% splenic suspension used.

Exper. 13—Amnionic route through air cell.

Exper. 14—Inoculum (5 cc.) dropped on ca. m. after removing shell and membrane over air cell. Original inoculum added frequently.

Exper. 15—Barnet's technique employed. Original inoculum added frequently.

Exper. 16—After removal of shell and shell membrane over air cell, 0.5 cc. on ca.m. Egg sealed with wide Scotch tape. Original inoculum added frequently.

Exper. 17—Injection of 0.03 cc. Blood of chick harvested and injected iv.

next colt in the series. The intranasal method of inoculation was used in the second to sixteenth passages, inclusive. The virus suspension, up to and including the fifteenth serial passage, consisted of nasal mucosa, lymph nodes, lung, and spleen ground in a glass mortar with alundum and suspended in defibrinated blood, preinfection serum, and small amounts of nutrient broth. Beyond the fifteenth serial passage, all colts were inoculated intravenously with finely ground infective spleen suspended in buffered saline solution containing 20 per cent preinfection horse serum. In the second to ninth serial passages, tissues were stored in 50 per cent glycerin solution (buffered at pH 7.0) in an electric refrigerator at 0 to 5 C. Materials were frozen and stored without preservative in the dry ice cabinet at –70 C. from the tenth passage onward.

Each experimental horse in table 2 is indicated by a circle. Each circle filled in with black represents 1 horse which became infected with typical equine influenza, and the numeral at the right of the black circle indictates the incubation period in days. It will be seen that some failures were experienced in attempts to infect horses which later proved to be fully susceptible. This was usually the result of loss of virulence in materials stored in buffered glycerin solution. Such failures were not experienced after the dry ice cabinet was used to store infective materials.

Neutralization of this virus strain with convalescent equine serum was demonstrated in the thirteenth serial passage. The procedure of this experiment will be described later in this report.

Difficulty was experienced in the experimental transmission of equine influenza between living horses. Nasal washings, although occasionally infective, were often nonvirulent, but finely ground suspensions of nasal and turbinate mucosae were much more regularly virulent. Blood was usually infective, but numerous transmission failures were experienced when naturally infected horses were used as the source of virus. Study of natural cases revealed that many animals experienced a transient fever, without other symptoms, one or more days before the appearance of typical fever and symptoms. The blood was regularly infective *only* within a few hours of the onset of this initial fever and often did not contain virus at any other time. It was, therefore, necessary to start recording temperatures several days prior to exposure to determine the individual animal's normal temperature range from which this initial rise could be readily detected.

The virus was found to persist in the epithelium of the upper respiratory tract for longer periods during the course of the disease. Here, however, bacterial contamination was usually present. It was found that the spleen contained appreciable amounts of virus in the earliest stages of the disease. Splenic tissues always were found to contain virus when collected *within a few hours* after the first appearance of fever. Suspensions of infective spleen were most often used as a source of virus.

NEUTRALIZATION OF THE VIRUS

It was found possible to neutralize the virus of equine influenza with convalescent or immune serum from horses. This was demonstrated in one test in the following manner. A virus suspension was prepared from the nasal mucosae of an infected colt by grinding the tissue finely and diluting it to 10 per cent by weight in buffered saline solution. Ten cc. of immune serum, collected from a horse on the thirteenth day of convalescence from equine influenza, was mixed with 10 cc. of this virus preparation, held at 4 C. for two and one-half hours, then administered intranasally to a susceptible colt. An equal amount of the same virus suspension was set up against normal (preinfection) colt serum administered to another susceptible colt.

The colt which received the virus-immune serum suspension remained well until twenty-six days later, at which time it was given an intranasal challenge dose of virus. Typical equine influenza followed on the third day after the challenge dose. In contrast, the colt which received the virus-preinfection serum mixture contracted equine influenza on the fifth day following instillation.

This test indicated the specificity of the virus under study and provided additional evidence as to its identity. This test was repeated later with comparable results.

THE INFECTIVE TITER OF THE VIRUS

It was not possible to titrate the virus of equine influenza as frequently as desired because of the scarcity of experimental horses; however, one such test will be described.

The inoculum consisted of a bacteria-free suspension from the eighteenth passage colt spleen, suspended in buffered saline to which 20 per cent preinfection horse serum had been added. Each colt received 20 cc. of a diluted suspension intravenously. One colt which received a 10^{-1} dilution began to have fever and symptoms on the third day; a second colt received a 10^{-2} dilution and started showing symptoms on the second day; a third colt received a dilution of 10^{-3} and remained well until given a challenge inoculation, at which time it proved susceptible.

This relatively low infective titer was a cause of concern and added to the difficulty of handling the virus.

ATTEMPTS TO CULTIVATE THE VIRUS

Two general methods were followed in our

endeavor to cultivate the virus of equine influenza—serial passage through embryonated chicken eggs and tissue cultures. In the chicken embryo experiments, standard methods of inoculation were employed as well as numerous modified techniques. Yolk sac and amnionic inoculations were usually made through the air cell. Techniques described by Burnet[2,3] were used for the most part in inoculating the chorioallantoic membrane (ca. m.), and the method reported by Eichorn[4] was used for intravenous inoculations. For each method of egg inoculation employed, such factors as age of the embryos, duration of incubation, and the character of the original and transfer inoculums were changed from time to time to exhaust the possibilities for growth by each method.

Table 3 has been prepared to simplify the presentation of the large amount of data accumulated concerning these efforts to grow the virus in the chicken embryo. An examination of this table will reveal that all attempts to cultivate the virus in the chicken embryo were unsuccessful.

The second general method was to cultivate the virus in tissue culture using the medium described by Sanders.[5] Four experiments were conducted, two of which were carried for ten serial passages, one for two, and another for four. Defibrinated blood drawn during the first day of influenza was used as inoculum for two series, and bronchial exudate collected during the first day of influenza and nasal washings from animals in the same stage were used to initiate the other two series. The cultures were incubated for four days in two series, three to five days in one, and four to eleven days in one. Eight flasks were inoculated, half of which were incubated at 37.5 C., the other half at room temperature. Presence of the virus could not be demonstrated in any of these series upon animal inoculation. The experiments were therefore terminated.

SYMPTOMS AND LESIONS IN EXPERIMENTALLY INDUCED INFLUENZA IN HORSES

Complete autopsy examinations were performed on all horses with natural and experimentally induced equine influenza, regardless of whether the animal died as a result of the disease or was killed to further the investigation. In each instance, the gross examination was followed by microscopic study of sections from all important tissues. The gross and microscopic lesions of animals with natural and experimental equine influenza have been described in a previous report.[6] Since the symptoms and lesions of the experimentally induced diseases are often mild in character and easily overlooked, particularly careful pathologic studies were carried out on horses experimentally infected. It was necessary to sacrifice most horses early in the course of the disease whenever virus was to be collected, therefore, in only a few instances, was this experimental disease allowed to run its entire course.

Differences were noted between the clinical character of the natural disease and that which was experimentally induced. These differences were only in degree of severity, however, since the tissues involved and type of pathologic lesion were similar. The experimental cases observed in this study were most like uncomplicated, natural cases of the disease. This feature and the great diversity[7]

TABLE 4—Symptoms and Lesions in Horses Following Intranasal Instillation of Equine Influenza Virus

Serial passage of virus	Incubation period (days)	Onset to death (days)	Symptoms		Gross and microscopic lesions				
					Nasal mucosa		Lung	Lymphnodes	
			fever	rhinitis	edema	petechiae	interstitial-pneumonitis	edema	petechiae
1	6	3	+	+	+	+	+	+	+
2	4	2	+	+	+	0	+	+	+
3	2	3	+	+	+	0	+	0	0
4	7	2	+	+	+	+	+	+	+
5	2	16	+	0	+	0	+	0	0
5	6	2	+	+	+	+	+	0	0
6	3	4	+	0	+	+	+	0	0
7	6	3	+	+	+	+	+	0	0
7	10	3	+	0	+	+	+	0	0
8	6	3	+	+	+	+	0	+	+
9	8	1	+	+	+	+	0	0	0
9	9	6	+	+	+	+	0	+	0
10	3	1	+	+	+	+	+	0	0
11	4	2	+	+	+	+	+	+	0
12	7	2	+	+	+	0	+	+	+
13	5	2	+	+	+	0	0	+	+
14	3	3	+	+	+	0	+	+	+
15	3	0	+	+	+	0	0	+	+

+ = present; 0 = absent.

of symptoms seen in equine influenza make it desirable to consider all possible data in order to be certain that the experimental disease was actually equine influenza.

A summary of the principal findings in 18 young horses which became infected with equine influenza following intranasal instillation of infected tissues is presented in table 4. The inoculum in most of these cases consisted of a suspension of finely ground nasal and turbinate mucosa, prepared as described in the section on materials and methods. These suspensions contained the equine influenza virus and occasionally bacterial organisms, chiefly *beta* hemolytic streptococci, which play a part in the severe natural disease and are constantly found in the upper respiratory tract in complicated cases. The incubation period varied from two to ten days in this series, indicating that the virus concentration in each inoculum varied in titer. Since most of these animals were destroyed within a few days after symptoms appeared, the lesions were minimal and easily overlooked. Fever was a constant symptom and varied from 103 F. to 105 F. Rhinitis started as acute congestion of the nasal mucosa, seen best on the Schneiderian membrane, followed by a catarrhal discharge which in a few cases became mucopurulent. Transient leucopenia was observed in some of the experimental cases, occurring in the earliest stages in much the same way as reported in the naturally acquired disease.[6]

Edema and congestion of the nasal mucosa were constant findings at postmortem examination. Petechial hemorrhages, sharply demarcated, were often seen in the mucosa of various parts of the nasal cavity. Erosion of the nasal epithelium was detected in some cases by microscopic examination, and lymphocytic and purulent infiltrations were occasionally seen in the underlying stroma. The lesions in the lungs were not often extensive and usually consisted of small, patchy areas of prune colored consolidation a few centimeters in diameter. These consolidated areas frequently followed the course of interlobular septums and were most common in the apical part of the lung. Microscopically, these lesions consisted of edema fluid richly mixed with lymphocytes, and occasionally neutrophils, and had a distinct tendency to involve the septums and interalveolar stroma.

Edema, congestion, and petechiae were seen in the lymph nodes of the head (submaxillary, parapharyngeal) and occasionally in those of the mediastinum. The lesions in these experimental cases were entirely consistent with those seen in the naturally acquired disease.

VIABILITY OF THE VIRUS

The viability of the virus was found to be adversely affected by several factors. Heat, fluctuation in pH values, and ordinary physiologic saline solution, all have a deleterious effect upon the virus. The destruction of the virus by heat has been prevented by carrying out all operations at low temperatures. This was accomplished by keeping tissue grinders in crushed ice, using cold diluent in the preparation of virus supensions, and immediately storing the suspension in the dry ice chest. The most satisfactory diluent was found to be the buffered (pH 7.2) physiologic saline solution to which 20 per cent preinfection horse serum had been added.

It will be seen from table 5 that the virus gradually lost virulence when stored in buffered glycerin at –4 C. Infection was produced in one colt after sixty-nine such days of storage, but failure resulted in another trial in which the virus had been stored for sixty-seven days. Virulence was maintained very well in tissues stored in the dry ice cabinet at –70 C. Full virulence was maintained under these conditions for twenty and one-half months in one instance, although in one other trial, after thirty-one months turbinate material was avirulent.

Living virus was found in the splenic tissues which had been frozen, desiccated, stored at –70 C. for fifty-four days, then at room temperature for twenty-four days and 119 days, respectively. Desiccated virus material in another trial survived for at least twenty-nine days when kept at 4 C.

It was not possible to determine the maximum survival time of the desiccated virus under all conditions, and the virus may possibly be kept virulent for much longer periods than these tests indicate.

IMMUNIZATION EXPERIMENTS

Natural Immunity to Experimental Infection. — Early in the course of experiments on the transmission of equine influenza, it was observed that young animals are usually susceptible, but the older animals are rarely affected. This, of course, was expected since an attack of the disease produces an immunity in most cases, although reinfection under natural conditions has been reported.[7] It was considered of value to study this immunity of older animals to experimental infection by inoculating a group of mature horses with the virus. Therefore, 10 horses, varying in age from 10 to 19 years, were given 10 cc. of a 10 per cent infective spleen suspension intravenously and observed for a subsequent period of ten days. Symptoms occurred in only 1 of these horses in which an atypical thermal reaction was observed on the fifth day. This horse was sacrificed and the spleen harvested, but suspensions prepared from this tissue proved to be aviru-

lent. There was consequently some question as to whether this was a case of experimental influenza.

This work indicates that the immunity of older horses, presumably as a result of exposure to the natural disease, is usually sufficient to protect them against experimental exposure to infective doses of the virus. This, of course, is further evidence of the relationship of the natural and experimental infections. Age is not the only factor in this immunity, however, since in another experiment a 17-year-old mare which had been on one farm all her life proved to be fully susceptible.

Bacterins and Killed Virus Vaccines.—Bacterins and killed virus suspensions were tested for immunizing properties shortly after the project was initiated. The bacterins were prepared, using streptococci isolated from fatal cases of equine influenzal pneumonia and transferred on mediums as few times as possible to prevent changes in antigenicity which might occur as the result of prolonged artificial culture. A few lots of nonvirulent virus vaccine were prepared by adding either formalin or phenol to defibrinated blood collected from horses with acute equine influenza. Splenic tissue vaccines inactivated with 0.5 per cent formalin for seventy-two hours at 20 C. likewise were prepared. In trials conducted during an extensive enzoötic of equine influenza, neither these bacterins nor the tissue vaccines proved to have any immunizing value against natural infection.

Living Virus Vaccine.—The isolation of the virus and its maintenance by colt to colt passage of splenic suspensions provided a virus strain which could be used in a vaccine, the antigenic potency of which was dependent upon the presence of live virus. Strain 183 virus material was selected from the nineteenth and subsequent colt passages when it was proved to be of a low virulence, and its effects could be predicted with reasonable assurance. This vaccine was kept in the frozen state at –70 C. and was also desiccated. The frozen tissue vaccine was given a trial under field conditions at Fort Robinson Q.M. Depot (Remount), Fort Robinson, Neb. The desiccated vaccine was tested for viability in susceptible horses in the laboratory but has not undergone any field trials. The object of desiccation is to permit shipment and handling of the vaccine, and the results of its use should not be significantly different from those of the frozen vaccine. The field immunization trials instituted early in 1946 at Fort Robinson were designed to determine the immunologic value of living influenza virus in splenic suspensions. The general plan used in these trials was as follows.

(1) The colts to be vaccinated in the experiment were isolated from the controls and all other susceptible animals immediately preceding vaccination, and this isolation continued until no further symptoms were exhibited by animals in any of the groups. They were returned to their stables only after it was reasonably certain that they were free of infection.

(2) Preinfection temperatures were taken for three days, during which period, preinfection serums were collected, and the animals were observed daily for signs of illness.

(3) The colts were inoculated with varying dosages of the virus by different routes in an effort to determine the optimum infective dose and the best route of inoculation. Some were inoculated intravenously, others subcutaneously.

(4) Records of temperatures and symptoms were continued twice daily after inoculation. Each colt was kept in a box stall and thus not allowed to exercise while fever was manifest.

(5) Immune serums were collected at varying intervals subsequent to cessation of symptoms.

(6) It was planned to keep detailed records of future outbreaks of influenza in all groups of depot-raised animals in order to compare the incidence and severity in vaccinated animals and unvaccinated controls.

This sixth step of the program could not be carried out as originally planned, because all but 12 of the colts were sold by the Army in the latter part of 1946. It was, therefore, necessary to write each purchaser of the colts to obtain the subsequent history. Each owner was requested to indicate whether his colts had been in contact with clinical cases of shipping fever; whether they were shipped long distances by rail or had been in public stockyards at any time; and whether symptoms of influenza or shipping fever had been observed. Considerable information was obtained by this means, as 62 of the 83 new owners answered, but in many cases the information was incomplete. It was not always possible to know from data furnished by the new owners whether the animal had been exposed to the disease except in those instances in which an outbreak was encountered or in which the animal in question actually developed the disease. It could be reasonably assumed that each animal which was shipped long distances by rail or handled through public stockyards had been exposed, since experience indicates that the disease is very frequently encountered under these conditions. The test would have been much more conclusive if all of the animals had been exposed in the same outbreak.

In this experiment, 95 horses were used; 51 were vaccinated and 44 were controls. The vaccinated group consisted of 15 yearlings, 11 2-year-olds, and 25 3-year-olds; the control

group was made up of 29 3-year-olds, 8 2-year-olds, and 7 yearlings. The results are outlined in table 6. It will be seen that for 16 animals in the vaccinated and 6 in the control group sufficient information was obtained to determine whether exposure and infection had taken place. None of the vaccinated animals developed the disease, but 3 of the controls did. The probability of such a result arising by chance is .013, indicating a significant difference in susceptibility between the two groups.

A point of particular interest in the use of this living virus as a vaccine is its effect upon the animal at the time of vaccination. The results were particularly encouraging in this respect since no unfavorable reactions were produced in any of the 51 colts which received the virus. A transient elevation of temperature, starting on the third day, was the only constant symptom observed. Moderate depression, transitory inappetence, and mild catarrhal rhinitis were observed in a very few animals. None of these signs was severe, and most would have been overlooked had the animal not been under close observation. It must be emphasized, however, that vaccination was carried out under highly favorable conditions, since no virulent secondary infection was present and the colts were protected from the elements, allowed adequate rest, and liberally fed. Under conditions similar to those outlined in this experiment, this vaccine appears to be a safe agent.

TABLE 6—Effect of Vaccination on Immunity of Colts to Natural Infection of Equine Influenza

Group	Subsequent history known			Subsequent history unknown
	I.	S.	T.	Total
Vaccinated ..... (51 colts)	16	0	16	35
Control ..... (44 colts)	3	3	6	38
Totals	19	3	22	73

I = immune; S = susceptible; T = total.

Approximately one year after the original vaccination, 12 of the young horses used in this experiment still remained under Army control. It was therefore possible to expose these animals to influenza virus by intravenous injection. Eight of these horses had been vaccinated as yearlings the preceding year, and 4 had been in the control group. After the challenge inoculation, 3 of the 8 vaccinated colts exhibited a mild febrile reaction, and 5 showed no symptoms at all. The 4 animals of this group which had not received any vaccine the preceding year all manifested

TABLE 5—Viability of the Virus of Equine Influenza

Trial No.	Tissue	Stored (how)	Stored (days)	Infective to colts
1	Nasal mucosae, tracheal washings* (1)	Refrig. at 4 C.	37	Yes
2	Nasal mucosae, lung*	Buffered glycerin at —4 C.	69	Yes
3	Lung, nasal mucosae	Buffered glycerin at 0 C.	67	No
4	Lung, blood, nasal mucosae*	Dry ice at —70 C.	15	Yes
5	Nasal mucosae	Dry ice at —70 C.	127	Yes
6	Nasal mucosae	Dry ice at —70 C.	256	Yes
7	Nasal mucosae	Dry ice at —70 C.	209	Yes
8	Spleen	Dry ice at —70 C.	31 mo.+	No
9	Defibrinated blood	Dry ice at —70 C.	11	Yes
10	Defibrinated blood	Dry ice at —70 C.	24	Yes
11	Defibrinated blood	Dry ice at —70 C.	24	Yes
12	Spleen	Dry ice at —70 C.	265 (8 mo.+)	Yes
13	Spleen	Dry ice at —70 C.	615 (20½ mo.)	Yes
14	Spleen	Dry ice at —70 C.	147	Yes
15	Spleen	Desiccated; —70 C., 54 days; 21 C. 24 days	78	Yes
16		Desiccated; —70 C., 54 days; 21 C. 119 days	173	Yes
17		Desiccated at 4 C.	29	Yes

*Pooled when tested.

febrile symptoms following experimental exposure. These 12 animals are included in table 6 but not elsewhere, since this table covers only the results of natural exposure.

In the course of various other experiments, it had been observed that all colts were solidly immune to reinfection with splenic virus suspensions during the first two months following an original febrile reaction. It is apparent that in yearling colts this immunity may not be complete after the lapse of a year.

SUMMARY

This paper is the outcome of investigations on equine influenza and its complications conducted in the Veterinary Research Laboratory while located at Front Royal, Va., and Fort Robinson, Neb., during the years 1939 to 1947, inclusive. The materials and methods developed by experience with this problem are described in detail. The virus of equine influenza could not be adapted to ordinary laboratory animals (ferrets, mice, rabbits, cotton rats, calves, rats, swine, cats, guinea pigs, and hamsters) hence was maintained by serial passage in young horses. One strain of virus has been maintained since 1941 by serial passage through colts alternated with storage of tissues in a dry ice cabinet at –70 C. Neutralization of the virus with convalescent serum was demonstrated, using colts as test animals. Attempts to cultivate the virus in developing chicken embryos or in tissue cultures were unsuccessful. The symptoms and pathologic lesions in experimental cases were found to approximate closely those of the mild form of the naturally acquired disease.

It was found possible to preserve the virus in equine tissues for as long as twenty and one-half months by storage in the dry ice chest at –70 C. The virus also survived desiccation and subsequent storage at 21 C. or 4 C. for at least 119 days. The virus was demonstrated in blood, serum, nasal washings, and nasal or turbinate mucosae, but the preferred source was the spleen, provided it was collected in the earliest part of the febrile stage of the disease. Parenteral injection of infective spleen suspensions of strain 183 virus, which is of low virulence, resulted in a mild febrile response followed by an immunity to further inoculations. One trial indicated that this vaccine has promise in immunizing against natural infection, although further tests are necessary. A desiccated virus vaccine was also prepared which may prove of value in immunization against the virus of equine influenza.

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Water-in-Oil Emulsions for Delaying Absorption of Penicillin in Horses

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A NUMBER of vehicles for preparing water-in-oil emulsions of penicillin for intramuscular administration have been prepared after the method proposed by Freund and Thompson.[5] The successful use of penicillin emulsions for treatment of gonorrhea and syphilis in human patients by single, daily, intramuscular injections has been reported.[6,7] On the other hand, a report by Ory, *et al.*[8] indicates that a water-in-oil emulsion used in their study was not superior to an equal volume of saline in maintaining serum levels of penicillin in human subjects. In a comparative study on horses and sheep, a water-in-oil and wax vehicle containing 2 per cent white wax in peanut oil caused no significant prolongation of the detectable blood level period over that obtained from use of saline solutions.[2]

MATERIAL AND METHODS

Normal horses were used in all dosage trials. Ages of the animals varied considerably, including suckling foals, weanlings, yearlings, and adult mares several years of age. Injections were made intramuscularly in the long head of the triceps and in the muscles of the neck. The penicillin* was the potassium salt of crystalline G and was dissolved in saline before emulsifying with the oil vehicle. One product labeled penol diluent,** containing oxycholesterol in vegetable oil, was combined in the proportion of 3.0 cc. of penol with 100,000 units of penicillin dissolved in 1.0 cc. of saline. Another product labeled pendil-improved formula,† containing cholesterol derivative 11 parts, and peanut oil 20 parts W/V, with beeswax 2 per cent W/V, was combined in various ways with penicillin dissolved in saline. In one series of trials, pendil was combined at the rate of 3.0 cc. with 100,000 units of pencillin contained in 1.0 cc. of saline. For another series of trials, the product was combined in proportion of 1.5 cc. with 1.0 cc. of saline containing 100,000 units of penicillin. In a third series, the proportion was 3.0 cc. of pendil to 1.0 cc. of saline containing 200,000 units of penicillin. The material for injection was prepared by hand shaking until a uniform emulsion was obtained.

For comparative purposes, a dose of 500 units per pound of body weight was used for all animals and for each combination of vehicle and penicillin solution. Doses were accurately measured in glass syringes. The entire volume of each dose was injected at one site. Except for modification of the penicillin concentration in the control series, the method for estimating the serum levels was the same as previously reported.[1] The determinations were made on samples obtained from the jugular vein at intervals of one or two hours following injection.

RESULTS

Seven serum level determinations were made with the penol diluent using doses of 500 units per pound of body weight. The emulsion, perpared with 3.0 cc. penol diluent and 100,000 units of penicillin in 1.0 cc. of saline, contained 25,000 units per cubic centimeter. In five trials, the duration of the measurable blood level was four hours, and in two trials the duration was three hours. The serum concentration in samples of blood obtained at fifteen and thirty minutes following injection, 0.32 to 1.28 units per cubic centimenter, indicate that absorption was not significantly retarded. Detailed results of each trial are given in table 1.

In the combination of 3.0 cc. with 1.0 cc. of saline containing 100,000 units of penicillin, pendil was administered to 10 horses. For 4 animals, the determinations were terminated at the eighth hour, with a penicillin concentration of 0.015 unit per cubic centimeter being present in the serum in three trials. Zero levels were obtained in 1 animal at the sixth hour, 1

The investigation reported in this paper is in connection with a project by the Kentucky Agricultural Experiment Station and is published by permission of the director.

*The penicillin used was supplied by Schenley Laboratories, Inc., New York.

**The penol diluent was supplied by the Haver-Glover Laboratories, Kansas City, Missouri.

†The pendil was supplied by Endo Products, Inc., Richmond Hill, New York.

TABLE 1—Penol*-500 Units per Pound Intramuscularly in Horses

Weight (lb.)	Total dose (units)	Units of penicillin/cc. of serum at specified intervals following injection in hours							
		.25	.50	1	2	3	4	5	6
1,240	620,000	1.28	1.28	0.64	0.16	0.04	0.015	0.00	0.00
880	440,000	0.64	0.64	0.32	0.16	0.08	0.015	0.00	0.00
980	490,000	0.32	0.64	0.32	0.32	0.16	0.02	0.00	0.00
1,260	630,000	1.28	1.28	0.64	0.16	0.04	0.015	0.00	0.00
1,100	550,000	1.28	0.64	0.32	0.08	0.04	0.015	0.00	0.00
260	130,000	1.28	0.64	0.32	0.08	0.02	0.00	0.00	0.00
150	75,000	0.64	0.32	0.08	0.015	0.015	0.00	0.00	0.00

*Haver-Glover Laboratories, Kansas City, Missouri—100,000 units of penicillin † in 1.0 cc. saline emulsified in 3.0 cc. penol.
†Potassium salt of crystalline penicillin G—Schenley Laboratories, Inc., New York.

TABLE 2—Pendil* 500 Units per Pound Intramuscularly in Horses

Wt. (lb.)	Total dose (units)	Units of penicillin/cc. of serum at specified time intervals following injection in hours													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
900	450,000	0.04	0.02	0.015	0.015	0.02	0.02	0.02	0.015	...	...	...	...	...	...
1,100	550,000	0.32	0.08	0.04	0.02	0.015	0.015	0.00	0.015	...	...	...	...	...	...
260	130,000	0.32	0.08	0.02	0.00	0.02	0.015	0.02	0.015	...	...	...	...	...	...
150	75,000	0.04	0.02	0.02	0.02	0.04	0.02	0.04	0.00	...	...	...	...	...	...
900	450,000	...	0.16	0.08	0.04	0.02	0.02	0.02	0.015	0.015	0.00	0.00	0.00	0.00	0.00
1,260	630,000	...	0.32	0.32	0.16	0.04	0.02	0.02	0.02	0.015	0.02	0.015	0.015	0.00	0.00
880	440,000	...	0.32	0.08	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
980	490,000	...	0.32	0.16	0.04	0.02	0.02	0.02	0.02	0.02	0.015	0.00	0.00	0.00	0.00
1,240	620,000	...	0.64	0.32	0.32	0.16	0.04	0.02	0.015	0.015	0.00	0.00	0.00	0.00	0.00
1,100	555,000	...	0.64	0.32	0.04	0.02	0.015	0.015	0.015	0.015	0.00	0.00	0.00	0.00	0.00

*Endo Products, Inc., Richmond Hill, New York—100,000 units of penicillin † in 1.0 cc. saline emulsified in 3.0 cc. pendil.
†Potassium salt of crystalline penicillin G—Schenley Laboratories, Inc., New York.

TABLE 3—Pendil* 500 Units per Pound Intramuscularly in Horses

Weight (lb.)	Total dose (units)	Units of penicillin/cc. of serum at specified intervals following injection in hours									
		1	2	4	6	7	8	9	10	11	12
1,240	620,000	0.32	0.16	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
340	170,000	1.28	0.64	0.02	0.00	0.00	0.00	0.00	0.00	0.00	0.00
980	490,000	0.64	0.64	0.08	0.015	0.00	0.00	0.00	0.00	0.00	0.00
120	110,000	1.28	0.08	0.015	0.00	0.00	0.00	0.00	0.00	0.00	0.00
850	425,000	0.64	0.32	0.04	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1,000	500,000	0.64	0.32	0.16	0.015	0.00	0.00	0.00	0.00	0.00	0.00

*Endo products, Inc., Richmond Hill, New York—100,000 units penicillin † in 1.0 cc. saline emulsified in 1.5 cc. pendil.
†Potassium salt of crystalline penicillin G—Schenley Laboratories, Inc., New York.

at the eighth hour, 3 at the tenth hour, 1 at the eleventh hour, and 1 at the thirteenth hour. In one trial, a zero level occurred at the seventh hour with recurrence of a measurable concentration at the eighth hour. In another trial, a zero level was obtained at the fourth hour with reappearance of a measurable quantity at the fifth hour. Considerable variation in serum concentration was observed on the first and second hours following injection. The concentrations at the first hour were 0.04 and 0.32 unit per cubic centimeter in two trials each. At the second hour, the concentration varied from 0.02 to 0.64 unit per cubic centimeter of serum for different animals. Except for concentrations of 0.16 and 0.32 unit per cubic centimeter at the fourth hour of two trials and 0.16 unit per cubic centimeter at the fifth hour of one trial, the concentrations were fairly uniform from the third hour to the end of the detectable blood level period, varying from 0.015 to 0.04 unit per cubic centimeter, table 2.

For six dosage trials, pendil was combined in proportion of 1.5 cc. with 1.0 cc. of saline containing 100,000 units of penicillin. In this series of trials, the third and fifth hour periods were omitted. Penicillin was present in detectable quantity at the fourth hour, but not at the sixth in four trials. In two trials, a measurable quantity was present at the sixth hour with zero levels occurring at the seventh hour. Penicillin was readily absorbed from the emulsion as indicated by serum levels of 0.32, 0.64, and 1.28 units per cubic centimeter at the first hour following injection. The serum concentration fell rapidly from the first hour to the zero level periods at the sixth and seventh hours. Results of the individual determinations are shown in table 3.

With 200,000 units of penicillin in 1.0 cc. of saline combined with 3.0 cc. of pendil, measurable serum concentrations were present for five hours in one trial, six hours in four trials, and seven hours in one trial. Penicillin concentrations in measurable quantity did not recur in the serum at any time following the initial zero level periods. The highest serum levels, 0.32 to 0.64 unit per cubic centimeter, were present at the first hour following injection. In general, a gradual fall in serum concentration was observed throughout the detectable blood level period, table 4.

DISCUSSION

With the penol diluent, the results were slightly superior to those observed previously with a water-in-oil plus wax vehicle, in which the period of detectable blood level did not exceed three hours for horses nor five hours for sheep.[2] The longer duration of the blood level in sheep appears to be of no significance in comparison with the penol diluent, as sheep have been found to maintain measurable blood levels for one to two hours longer than horses following doses of 500 units per pound in saline solution intramuscularly.[3] Using aqueous solutions of penicillin by intramuscular injection in horses, a dose of 500 units per pound has maintained detectable blood levels for periods of two to three and one-half hours.[1] With this dose, the aqueous solutions produced serum concentrations of 0.62 and 1.24 units per cubic centimeter

TABLE 4—PENDIL* 500 Units per Pound Intramuscularly in Horses

Weight (lb.)	Total dose (units)	Units of penicillin/cc. of serum at specified intervals following injection in hours											
		1	2	3	4	5	6	7	8	9	10	11	12
480	240,000	0.32	0.16	0.08	0.08	0.015	0.00	0.00	0.00	0.00	0.00	0.00	0.00
420	210,000	0.32	0.16	0.08	0.04	0.02	0.02	0.00	0.00	0.00	0.00	0.00	0.00
430	215,000	0.64	0.32	0.08	0.02	0.015	0.015	0.00	0.00	0.00	0.00	0.00	0.00
410	205,000	0.32	0.16	0.08	0.08	0.08	0.08	0.00	0.00	0.00	0.00	0.00	0.00
310	155,000	0.32	0.32	0.16	0.16	0.08	0.04	0.015	0.00	0.00	0.00	0.00	0.00
520	260,000	0.32	0.16	0.08	0.08	0.04	0.02	0.00	0.00	0.00	0.00	0.00	0.00

*Endo Products, Inc., Richmond Hill, New York—200,000 units penicillin † in 1.0 cc. saline emulsified in 3.0 cc. pendil.
†Potassium salt of crystalline penicillin G—Schenley Laboratories, Inc., New York.

at twenty- and forty-minute periods following injection. With the penol diluent serum levels of 0.32, 0.64, and 1.28 units per cubic centimeter were obtained at quarter-hour and half-hour periods following injection. Evidently there was no significant delay in the initial absorption of penicillin from the emulsion. The duration of the detectable blood level for the penol diluent was one to two hours longer than obtained with aqueous solutions.

With the combination of 3.0 cc. of pendil with 100,000 units of penicillin in 1.0 cc. of saline, there was a significant delay in absorption of penicillin from the emulsion. The serum concentrations of two trials, 0.32 unit per cubic centimeter at one hour following injection, approximated those obtained with the penol diluent and aqueous solutions, while two others, 0.04 unit per cubic centimeter, were considerably less. Generally, the serum concentration at the two-hour period was higher than obtained with the penol diluent and aqueous solutions. The most significant evidence of delayed absorption from the pendil menstrum was the prolonged period of detectable blood level, which varied in most trials between eight and ten hours. With the same dosage of penicillin in units per pound of body weight, the pendil maintained measurable blood levels from one to seven hours longer than the penol diluent; from two to eight hours longer than the water-in-oil plus wax vehicle, previously investigated;[2] and from three to ten hours longer than penicillin in aqueous solution.[1] In general terms, with this combination, pendil, 3.0 cc., and penicillin, 100,000 units in 1.0 cc. of saline, the serum concentrations were comparable in duration in hours and concentration in units per cubic centimeter to those obtained with penicillin in oil and wax, 300,000 units per cubic centimeter, (Romansky formula);[4] however, the serum concentrations with pendil tended to run at higher levels during the first two hours than with the Romansky formula.

In this combination, the pendil emulsion, prepared with 3.0 cc. pendil and 100,000 units of penicillin in 1.0 cc. of saline, contained 25,000 units of penicillin per cubic centimeter. For a dose of 500 units per pound, a volume of 20.0 cc. is required for a 1,000-lb. animal. For the larger horses used in these trials, volumes of 17.6 to 26.2 cc. were required. This volume of material proved rather irritating to the tissues, resulting in significant swelling and soreness which persisted from four to six days following single intramuscular injections. The smaller doses of 3.2 and 5.0 cc. caused no undesirable reactions in the suckling colts. Since the material would have to be injected at intervals of eight hours to maintain a measurable concentration of penicillin in the serum, it appears that the irritation may prove too severe to permit use of the product for a course of treatment covering several days.

Following the irritation experienced with the combination discussed above, other proportions of pendil and penicillin in saline solution were studied. In order to reduce the volume of the dose, pendil was combined in the proportion of 1.5 cc. with 1.0 cc. of saline containing 100,000 units of penicillin, making an emulsion containing 40,000 units per cubic centimeter. With this combination, a dose of 12.5 cc. was required for a 1,000-lb. animal. The irritant effects resulting from the reduced volume of material were practically eliminated, but there was a marked reduction in ability of this emulsion to retard absorption of penicillin. The period of detectable blood level varied from four to six hours, being approximately four hours less than obtained with the suspension of 3.0 cc. pendil to 1.0 cc. of penicillin solution.

Further attempt to avoid the irritation of the large volume was made by combining 3.0 cc. of pendil with 1.0 cc. of solution containing 200,000 units of penicillin. In this combination, the emulsion contained 50,000 units per cubic centimeter, requiring a volume of 10.0 cc. for a 1,000-lb. animal. Again the irritant effects were avoided. The duration of the period of measurable blood level averaged six hours, approximately two to three hours less than the average obtained with the 3 to 1 emulsion containing 25,000 units per cubic centimeter.

To secure an efficiency in delaying absorption of penicillin comparable to that obtained with penicillin in oil and wax (Romansky formula),[4] it appears that pendil emulsions should contain at least 1 volume of penicillin solution to 3 volumes of pendil. Also it is apparent that the volume of emulsion administered by intramuscular injection must approximate 2.0 cc./100 lb. of body weight to produce blood levels comparable in duration to those derived from equivalent doses of penicillin in oil and wax, 300,000 units per cubic centimeter.[4] The irritant effect of this volume of emulsion raises doubt as to its adaptability to large animal practice, particularly in light horses, purebred cattle, and work-stock.

SUMMARY

The penol diluent was found to prolong detectable blood levels of penicillin for one to two hours longer than those obtained by intramuscular injection of penicillin in saline solution.

An emulsion prepared by combining 3.0 cc. of pendil with 1.0 cc. of saline containing 100,000 units of penicillin was found comparable to the Romansky formula with doses of 500 units per pound of body weight for each product. With this dose, 2.0 cc. of emulsion was required for each 100 lb. of body weight, and in large animals it caused considerable irritation of the tissues. Altering the proportions of pendil and solution to reduce the volume of the injections resulted in lowered efficiency of the product for delaying the absorption of penicillin.

Penicillin in oil and wax (Romansky formula) is considered a more suitable product for field use in large animal practice.

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Penicillin Therapy in Chronic Bovine Mastitis. II. Penicillin Levels in the Udder During Treatment

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EARLY WORK on the pharmacology of penicillin in the human and animal body stressed the rapidity with which this drug was eliminated from the body by way of the kidneys. Therefore, it was found necessary to use repeated administrations at intervals of four to eight hours in order to maintain an adequate blood level.

When penicillin became available for use in the treatment of bovine mastitis, it was not known how frequently the injections should be made in order to maintain a satisfactory level in the udder over a period of time. Some information on this phase of the problem seemed essential for most efficient administration.

LITERATURE

Schalm[5] tested the milk of 3 cows twenty-four hours after each of the udders had been infused with 50,000 Oxford units (O.U.) of calcium penicillin. He records 0.003 to 0.624 units per cubic centimeter of milk. In another trial, using 7 cows, the milk was assayed twelve hours following the infusion of 20,000 to 50,000 O.U. per quarter. Readings of 1.94 to 20 O.U. were recorded. In these trials, the amount of milk produced was correlated with penicillin levels. It was pointed out by Schalm that in high producing cows the levels of penicillin were lower than in cows producing less milk. Similarily, Murnane[3] injected 15,000 O.U. per quarter into the udders of cows in various stages of lactation and assayed the milk twelve hours later. Milk from cows in full lactation assayed 0.6 units per cubic centimeter, in middle lactation 1.2 to 5 units, cows in the "drying off" period 21 units, and 40 units in dry cows. Stevenson[7] reports on 1 cow producing 13 lb. of milk per day which had 14 units of penicillin per cubic centimeter of milk twelve hours after 25,000 units had been injected into the udder. Schofield[6] found an average of 0.44 O.U. in the milk twenty-four hours after infusing 25,000 O.U. and approximately 2.5 units twenty-four hours after 50,000 units were administered.

Thorp, Uhrik, and Straley[8] injected 10,000 to 200,000 O.U. into the udders in a series of lactating cows and made assays on the milk every two hours for eight hours. Substantial levels were maintained in the udder for eight hours. The higher dosages gave higher levels at the end of eight hours. The exact figures were not given, but from the graphs shown, the amounts ranged from 5 to 30 O.U. (estimated) where 10,000 units were injected, to 75 to 200 O.U. (estimated) when 200,000 O.U. were administered. A rapid decrease in the penicillin per cubic centimeter of milk was noted during the first two to four hours in all trials. Weirether, Jasper, and Petersen[9] report that after the infusion of 30,000 or more O.U., a level of 0.5 O.U. or more is present after twenty-four hours. These investigators also report that when penicillin is carried in large amounts of diluent, the concentration of penicillin per cubic centimeter increases temporarily because the liquid is quickly absorbed and leaves the penicillin in the udder. They also report 11 O.U. in the

TABLE I—Penicillin Assay Data on Case 1839, Average Daily Production 57.5 lb.

Date	Time		R.H.	L.H.	L.F.	R.F.
		Condition of quarters	*Sta. aureus*	neg.	neg.	neg.
5-27-47	4:00 p.m.	Injection O.U.	100,000	100,000	25,000	..
5-28-47	6:00 a.m.	Milk assay O.U. per cc.	38	38	8	0
		Injected O.U.	200,000	.....	.....	..
	4:30 p.m.	Milk assay foremilk	29	3.8	.5	0
		Strippings	22	.....	.....	..
		Injected O.U.	100,000	.....	.....	..
5-29-47	6:00 a.m.	Milk assay	18	0.4	0	..

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urine eight hours and 5 O.U. twenty-four hours after the administration of 40,000 O.U. in the udder. Schofield[6] reported pencillin levels of 5 and 15 O.U. per cubic centimeter at one-half and two hours, respectively, after

TABLE 2—Penicillin Content of Milk Drawn Immediately Before and Immediately After the Regular Milking Period

Case No.	Assay of foremilk (O.U./cc.)	Assay of strippings (O.U./cc.)
1839	29	22
2500	63	22
	18	30
1828	8	8
	13.5	13.5
	3.8	10
	6.3	8
	6.3	6.3
	18	10
2359	18	29
	22	18
12	22	29
	38	29
	50	38
	80	50
Ave.	26.4	21.7

100,000 units had been injected into two quarters. Eight units per cubic centimeter were present in the urine after six hours.

METHODS OF PROCEDURE

Samples of milk for assay were collected in sterile test tubes and held in the refrigerator until run. Usually 3 to 5 cc. were collected at the regular milking time just before repeat injections of the drug were made. Either foremilk or strippings was used and, in some cases, both were collected, depending upon the circumstances. The milk samples seldom were held more than eight hours before assays were made; many were made less than one hour after collection.

The agar cup assay technique patterned after Cholden[1] was followed throughout. The test organism used was *Staphylococcus aureus* 313 which had an average sensitivity of .03 to .06 O.U. The test strain was grown in nutrient broth for twenty-four hours at room temperature. Growth was more uniformly turbid at room temperature than at 37 C. Five cubic centimeters of the 24-hour culture were shaken with glass beads to give an even distribution of cells. This 5-cc. amount was used to inoculate 250 cc. of melted tryptose agar which had been cooled to a temperature of 45 to 48 C. This amount of seeded agar was used to pour 12 standard 100-mm. Petri plates, care being used to distribute the quantity as evenly as possible to each plate to insure uniform depth. Each plate contained three sterile rubber stoppers, 00 size, arranged equidistant from each other and from the side of the plate. After the agar had hardened around the stoppers, they were removed leaving an agar cup. The bottom of the cup was sealed with one drop of sterile, melted agar. It was found advantageous to chill the plates for a few hours in the refrigerator before using. If this were done, the zones of inhibition were more distinct. Then 0.2 cc. of the milk or saline dilutions of milk to be tested was placed in the cup and the plate incubated at 37 C. for eighteen to twenty-four hours. The width of each zone was measured in millimeters with the aid of a "penicillin reader."*

One unit standards were run with each batch of assays, and the amounts of penicillin in the milk were read from a standard curve. Urine samples were run using the technique described above.

RESULTS

Table 1 shows the differences in levels of penicillin in the milk when the same dose is given in an infected (right hind) and a normal quarter (left hind) of the same cow. Also, the difference in penicillin concentra-

*A Quebec colony counter modified by Fisher Scientific Co.

TABLE 3—Data on Three Cows Injected with Varying Doses of Sodium Penicillin Dissolved in Saline

Case	Quarter	Penicillin injected (O.U.)	Vehicle (cc.)	Assay of milk at 12 hr. (O.U.)	Milk secreted during 12 hr. (cc.)	Estim. of penicillin removed in milk (O.U.)	Assay of milk at 24 hr. (O.U.)	Temp. change 7 hr.**
12	R.H.	100,000	50	25	825	21,000	1.8	+1.7 F
	L.H.	100,000	50	33	825	27,000	1.35	
	L.F.	200,000	200	44	585	25,700	3.8	
	R.F.	200,000	10	65	660	43,000	1.6	
13	R.H.	100,000	50	29	100	2,900	2.9	+1.4 F
	L.H.	100,000	50	29	110	3,190	1.15	
	L.F.	25,000	12½	22	30	660	1.0	
	R.F.	25,000	12½	50	25	1,250	2.9	
14	R.H.*	100,000	50	18	730	13,000	.5	+1.0 F
	L.H.	100,000	50	29	525	15,200	1.0	
	L.F.	50,000	25	80	210	16,800	2.9	
	R.F.	50,000	25	29	230	6,760	2.3	

**Temperatures furnished by Dr. L. M. Jones, Department of Veterinary Pharmacology and Dr. John Arnold, Department of Veterinary Surgery.

tions in the milk after 100,000 units and 25,000 units were injected into two normal quarters, L.H. and L.F., of the same cow are compared. A reading of 38 units was obtained twelve hours after 100,000 units were administered and 8 units in the quarter receiving 25,000 units. Assays of the milk collected after twenty-four and thirty-six hours showed corresponding differences in penicillin levels, the 100,000 dose giving higher readings. The two rear quarters were given the same initial dose of 100,000 units with assays after twelve hours reading the same, 38 units. The R.H. quarter received two other injections at twelve-hour intervals while the L.H. received none. Milk collected twenty-four hours following the intial dose showed marked differences in penicillin levels, 29 units for the R.H. which had received two injections during the 24-hour period and 3.8 units for the L.H. which received only one injection during that period. Two attempts were made to detect penicillin in the milk from the R.F. quarter which was not injected, but none could be demonstrated.

Although the results presented in table 2 show no marked differences between foremilk and strippings, the average of foremilk is about 5 units higher, which would indicate that the distribution of penicillin in the udder is fairly uniform, at least after the udder has been filled with secretion.

A comparison of the data in table 3 shows only minor differences in the level of penicillin in the milk twelve hours after the injection of 100,000 O.U. into the rear quarters of all 3 cows. Three of these six quarters gave identical readings of 29 units per cubic centimeter of milk, the other three being 25, 33, and 18 units per cubic centimeter. The milk from the two quarters receiving 200,000 units gave somewhat higher values. One of these quarters received the 200,000 units in 200 cc. of diluent, while the other received the same dose in 10 cc. Some swelling was present at two and four hours after injection in the quarter receiving 200 cc. of liquid, while none appeared in the one receiving 10 cc. even though the quantity of penicillin was the same in both cases. This swelling was not detectable at eight, ten, or twelve hours following injection. In cases 13 and 14, the fore quarters were given doses of 25,000 and 50,000 units, respectively. Assays of milk from these quarters compared closely with those given two to four times as much. However, it will be noted that the volume of secretion from these quarters is less.

Calculation of the amount of penicillin in the milk twelve hours after injection would indicate that much less penicillin actually left the udder through absorption into the blood stream from the quarters producing more milk. Comparison of assays made at the 24-hour period shows only minor differences between the penicillin levels of the quarters regardless of the amount of penicillin injected or the volume of milk secreted.

A general temperature increase was noted seven hours after the penicillin administrations were given. These were rectal temperatures in which the average of three preinjection and two postinjection temperatures were compared with one taken seven hours after the penicillin injection. This would indicate some slight irritant properties of the material injected.

The summaries shown in table 4 are the results of 107 separate trials using 44 different quarters of 16 cows. With the exception of 3 cows, all were being treated for mastitis. The similarity of penicillin levels at either the twelve- or 24-hour periods after the injection of the various doses is quite striking. Average readings obtained on milk taken twelve hours after the injection of either 50,000 or 100,000 O.U. were identical. In these two instances, the number of trials is large enough to carry significance. There are no marked differences in the quantity of penicillin twenty-four hours after the infusion of 25,000 or 200,000 units.

Only five samples of urine were assayed for penicillin, and no attempt was made to study the rate or amount of penicillin excretion. It is of interest to compare these few deter-

TABLE 4—Penicillin Levels Present in the Milk Under Varying Schedules of Treatment

Hours between injection and testing	Dosage of penicillin (O.U.)	No. of trials	Milk assays range (O.U./cc.)	Ave. (O.U./cc.)
12	200,000	3	22 — 80	44.8
	100,000	32	3.8 — 50	19.4
	50,000	53	1.8 — 80	19.4
	25,000	3	8 — 50	26.7
24	200,000	2	1.6 — 3.8	2.7
	100,000	7	.5 — 3.8	1.8
	50,000	2	2.2 — 2.9	2.5
	25,000	3	.5 — 2.9	1.4
36	100,000	1	.4	.4
	25,000	1	0	0

minations with those reported by other investigators.

DISCUSSION

The penicillin levels reported in the preceeding tables compare with those given by Weirether, Jasper, and Petersen,[9] Stevenson,[7] and Thorp, Uhrik, and Straley.[8] The readings given in table 4 are somewhat higher than those reported by Schofield[6] and Schalm,[5] where comparable doses were administered. As Schalm[5] has pointed out, the concentration of penicillin remaining in the udder depends to some extent on the amount of secretion during the period. Therefore it is difficult to make direct comparisons unless all conditions are duplicated. Schofield[6] also states that "penicillin is variable in the rate at which it is absorbed from the udder." Reference to table 4 shows the extreme ranges of penicillin levels obtained in different quarters, given identical doses, although a majority were close to the average figure given.

Case 1839 presented in table 1 shows no difference in the rate of disappearance of penicillin from a normal quarter as compared to that of one with chronic infection. Two quarters of 2 cows, 2500 and 2537, which were treated during acute infection showed no difference in the rate of absorption from the infected quarters. The decline in penicillin level from 38 to 18 units in the R.H. quarter of cow 1839 despite continued injections is not explained. This was not a general occurrence in other cows treated similarly. Comparison of the milk assays on the rear quarters of this cow emphasizes the need for frequent administration of penicillin in the lactating cow, if a high level of the drug is to be maintained over a period of time.

No literature was available regarding the penicillin content of different portions of the milking or which was most representative of the quantity of penicillin in the udder. Therefore, a number of tests were made on the first 3 to 5 cc. of milk drawn from the quarter and compared with the level of penicillin in the last 3 to 5 cc. drawn. Table 2 shows that the penicillin content of some foremilk samples was higher, but in other cases strippings gave higher readings. Average levels were 26.4 O.U. for the foremilk and 21.7 O.U. for strippings on the 15 quarters tested. The foremilk contained an average of about 5 units more per cubic centimeter of milk, but indicated a fairly uniform distribution in the udder, which is quite logical since penicillin is soluble in water and would readily diffuse in the milk serum.

The assays of penicillin remaining in the milk of the 3 cows of table 3 are quite similar in all quarters. This is especially true at the 24-hour period after injection. If only these figures were seen, it would be difficult to predict which quarters received the 25,000 units and which the 200,000 units as the original injection. Likewise, it would be difficult at twelve hours to estimate the dose given. These observations would suggest that there may be "threshold levels" maintained in the udder which may be somewhat independent of dosage. It may further be suggested that within the limits of dosage used, the frequency of administration is much more important than the quantity of penicillin injected in maintaining substantial levels in the udder. Schalm[4] has suggested that larger doses of penicillin should be administered to cows in high production in order to maintain a high concentration of the drug in the milk. The data presented in table 3 do not uphold that

TABLE 5—Penicillin Assays on Urine Samples Taken During Treatment

Case No.	Amt. of penicillin per injection	No. of inj.	Amt. penicillin given before urine taken	Hours after last injection	Urine assay (O.U./cc.)
1839	100,000	3	300,000	14	2.9
1725	50,000	6	300,000	12½	3.8
1863	50,000	6	300,000	12½	2.9
13	100,000	2			
13	25,000	2	250,000	12	3.8
14	100,000	2			
14	50,000	2	300,000	12	10

recommendation. They show that much more penicillin actually leaves the udder in a low producing cow than in a high producing one, resulting in about the same level of penicillin in each case at twelve or twenty-four hours after the injection.

The temperature increases of 1 to 1.7 F. reported in table 3 show that there was undoubtedly some irritant substance in the penicillin or saline solution used. Thorp, Uhrik, and Straley[8] also observed some reactions and attributed them to pyrogenic substances in the saline solution. Petersen[3] has shown that saline is irritant when injected into the bovine mammary gland. It is possible that the mild reactions were due in part to the saline injected, although the exact cause of the temperature rise was not determined. The only quarter out of the 12 injected in which any swelling could be detected was the L.F. of cow 12 which received 200 cc. of a solution of saline and penicillin. These reactions were not in any way serious nor were they considered detrimental.

The results presented in table 5 show that penicillin was absorbed from the udder and eliminated in the urine. The levels obtained are near the ranges given by Schofield[6] and Weirether, Jasper, and Petersen,[9] although the dosages used and the time lapse after injection were not the same.

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Penicillin Therapy in Chronic Bovine Mastitis. III. Treatment of Mastitis

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THE TREATMENT of bovine mastitis has been the subject of many studies in years past. Almost every drug known to have therapeutic value has been tried one way or another in attempts to cure infections of the bovine udder. Need for an efficient therapeutic agent has become increasingly important to the dairyman and to the practicing veterinarian as more attention is being directed toward this important disease. It has been less than ten years since the first effective agent was used in this country. Since that time, a great deal of progress has been made in chemotherapy of mastitis. The chemicals used first, such as acridine dyes and silver oxide, although effective in selected cases, were irritant to the tissue and could be expected to successfully eliminate only the stroptococci from the udder. The dramatic and spectacular success of penicillin in human infections immediately raised the hopes of veterinarians and research workers in mastitis control, that probably this new drug would be the answer to the mastitis problem. These expectations are now only partially fulfilled after three years of experimentation.

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LITERATURE

The first published report on the use of penicillin in bovine mastitis was by Kakavas[1] who used both crude and partially purified sodium penicillin. Injections were made at 24-hour intervals and doses of 1,000 to 15,900 Oxford units were administered one to eight times. Sixteen of 27 Staphylococcus cases and 21 of 23 *Streptococcus agalactiae* infections treated with crude preparations were reported negative after treatment.

Considerable data on clinical trials with commercial penicillin have been compiled since the first report.[1-6, 8, 9, 11-18, 20] A majority of these reports, especially those dealing with the use of penicillin in streptococcic mastitis, are favorable, inasmuch as success is reported in about 60 to 100 per cent of the cases treated. The percentage of successful treatments is less with staphylococcic cases of mastitis. These range from 0 to about 80 per cent, with more of them below 50 per cent than above. These reports have led most workers to the general conclusion that staphylococcic infections are more difficult to treat than those caused by streptococci. They agree, however, that penicillin is the drug of choice for the treatment of both streptococcic and staphylococcic mastitis.

TABLE I—Summary of Penicillin Treatment and Tests Following Treatment

	Crude penicillin	Sodium penicillin	Totals
No. of quarters treated	56	66	122
No. of injections made	179	299	478
Ave. No. of injections per quarter	3.2	4.4	
Total units of penicillin used	2,127,100	13,515,000	15,642,100
Ave. units of penicillin per quarter	38,000	204,700	
Ave. No. of tests after treatment	4.1	2.7	
Ave. No. of weeks observed after treatment	18.1	10.3	

METHODS

Description of the Herd.—The observations reported here, on the value of penicillin, were made on the Iowa State College dairy herd. This herd is maintained at approximately 100 cows in milk the year around. It is composed of representatives of all the common dairy breeds, with Holstein-Friesians predominating. The husbandry of this herd is considerably above average. Machine milking, using a "combine milker" at twelve-hour intervals,

TABLE 2—Comparison of Crude and Sodium Penicillin Administered One to Twelve Times in Staphylococcic and Streptococcic Mastitis

	Result after treatment	Crude penicillin injections						Sodium penicillin injections					
		1 to 2		3 to 4		5 or more		1 to 2		3 to 4		5 or more	
		Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
Sta. aureus	No. of cases	1	8	10	9	3	4	2	1	17	15	3	2
	Per cent	22	78	53	47	43	57	67	33	53	47	60	40
Streptococci*	No. of cases	11	2	7	1			4	1	13	4	4	0
	Per cent	85	15	87.5	12.5			80	20	76	24	100	

*This includes *Str. agalactiae*, *Str. dysgalactiae*, and *Str. uberis*.

TABLE 3—Summary of All Treatments; Efficiency of Penicillin in Staphylococcus Aureaus Cases Compared with Streptococcic and Frequency of Injections in These Two Types

	Injections	1 to 2 24-hr. intervals		3 to 4 12-hr. intervals		5 or more 12-hr. intervals		Totals	
	Results of tests after treatment	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.
Sta. aureus	No. of cases	3	9	27	24	6	6	36	39
	Per cent	25	75	53	47	50	50	48	52
Streptococci	No. of cases	15	3	20	5	4	0	39	8
	Per cent	83	17	80	20	100	0	83	17

TABLE 4—Protocols of 2 Cases Showing Recovery in One and Improvement in the Other

	Case 2537			Case 1839		
Date	pH test	Catalase cc. gas	Cultures	pH test	Catalase cc. gas	Cultures
4-14-47	—	9	Str. dysgalactiae	+	12	*Sta. aureus*
4-21-47 to 4-23-47	150,000 units of penicillin, 3 injections 12 to 24-hr. intervals			300,000 units of penicillin, 4 injections 12-hr. intervals		
4-22-47				+	7	—
4-28-47	—	5	—	—	3½	*Sta. aureus*
5- 5-47	—	2	—	+	3	*Sta. aureus*
5-26-47	—	½	—	+	4	*Sta. aureus*

TABLE 5—Milk Production of 9 Representative Cases Before, During, and After Penicillin Therapy

Case No.	No. of quarters treated	Average daily milk production lb.* 7 days before treatment	7 days during treatment	7 days after treatment	Remarks
2380	1	11.1	6.9	6.3	Nearly dry
2383	2	40.2	39.2	39.7	
2156	2	54.7	53.2	48.7	
2500	1	25.9	15.5	25.6	Acute attack R.H. quarter
2537	2	22.9	22.7	33.7	Acute infection L.H. quarter
1836	2	53.9	53.1	58.9	
1839	3	58.2	61.3	55.0	
1828	4	55.4	54.8	54.4	
2061	4	44.1	43.5	40.2	

*From records of the Department of Dairy Husbandry, Iowa State College.

was employed during the period covered in this study. The milking sanitation is above average. Each udder is wiped with a separate chlorine-soaked cloth just prior to milking, and the teat cups are immersed in a steam bath heated to 170 F. for three to four minutes between cows.

A program of mastitis control was started in this herd in the summer of 1941. At this time, *Str. agalactiae* infection predominated with scattered cases caused by *Streptococcus uberis*, *Streptococcus dysgalactiae*, and *Staphylococcus aureus*. The program of mastitis control involved milking order, improvements in sanitation in milking, culling badly infected individuals, and treatment of suitable cases with acriflavine, silver oxide, tyrothricin and, later, sulfanilamide.

By the spring of 1944, three years later, only 1 case of *Str. agalactiae* infection remained in the herd. This case was among the first treated with penicillin, and the only four quarters of this type of infection treated and reported in this paper are from this cow. No infections caused by *Str. agalactiae* have appeared in the three years since this cow was treated.

The chemotherapeutic agents just mentioned were not effective in eliminating *Sta. aureus* infections, so a number of cows showing this type of infection remained, as well as a few *Str. uberis* and *Str. dysgalactiae* infections. Some new cases of each type have appeared since that time. These cases which were treated with penicillin comprise this part of the report.

Diagnostic Methods.—The diagnosis of the cases treated was based on chemical tests and the culture and identification of the causal organism. Clinical observation and physical examination were aids in a few instances.

The tests used were strip-cup, pH, Hotis; and catalase. These were accompanied by a blood agar culture of incubated samples from each quarter. Identification of the streptococci was made on the basis of biochemical reactions. *Sta. aureus* was identified by hemolytic and plasma-coagulating characteristics.

Preparation of Penicillin.—At the time this part of the work was begun (May, 1944), there were no published reports on the use of penicillin in domestic animals. Neither was there any commercial penicillin available for animal use. Therefore, the penicillin which was used at first was prepared in the laboratory, injected in the crude form, and dosages arbitrarily determined.

Crude penicillin was prepared by growing *Penicillium notatum* N.N.R.L. 1249•B21 on the surface of Czapek-Dox broth modified according to Moyer and Coghill.[10] The cultures were grown at 25 to 27 C. in 16-oz. bottles containing 100 cc. of broth. After nine to eleven days' incubation, the penicillin was "harvested" by pouring off the broth and filtering it, first through coarse paper, and then sterilizing it by passing it through a Mandler filter candle. Filtrates having a potency of 80 to 375 O.U. per cubic centimeter were obtained under these conditions. Twenty to 80 cc. of this crude penicillin were injected into the gland cistern two to four times at either twelve- or 24-hour intervals.

The commercial penicillin* used consisted of several different lots of sodium penicillin in the amorphous form. Some of the last cases were treated with a residue from the preparation of crystalline G fraction. This residue was composed of about 26 per cent K fraction and the remainder largely F fraction.

The penicillin was dissolved in sterile saline, distilled water, or 10 per cent lactose solution. Only a few cases were treated with distilled water or lactose solution as the diluent.

With few exceptions, the quantity of penicillin was given in 50 cc. of solution.

Injections were made using a 50-cc. glass syringe and a teat cannula, a separate cannula being used for each injection. After injection of the penicillin, the quarter was massaged to aid in distribution of the drug. Injections were made immediately after complete milking of the cow. In order to determine the effect of the injections, samples were taken for chemical cultural tests at various times. Usually tests were made at weekly intervals for several weeks after treatment, or in some cases only until the organism was recovered again from the quarter.

The tests employed in checking after treatment were the strip-cup, pH, catalase, and Hotis tests and blood agar cultures of incubated samples.

RESULTS

The results of administering penicillin to 53 cows with infection in 122 quarters are summarized in the tables.

The examination of table 1 shows that fewer injections were made and less penicillin used per quarter when crude filtrates were used, which was largely due to the short supply of the drug when it had to be made in the laboratory. These differences should be borne in mind when the results presented in table 2 are examined. Since the crude penicillin was used first, the number of tests after treatment and the period of observation after treatment were greater than with the sodium penicillin. Some quarters were examined eight to nine times after treatment. In a few cases, only one test was made following treatment, since upon the first test the organisms were found

*Furnished by Merck & Co., Rahway, N. J.

to persist in the udder. A majority were observed for three to four months after treatment. A few quarters were observed for over two years.

The results of the treatment of comparable cases of streptococcic and staphylococcic mastitis (table 2) show no significant differences in the efficiency of treatment with these two types of penicillin. The percentage of successful treatments of streptococcic cases is 80 to 85 per cent with either crude filtrates or sodium penicillin. Furthermore, it is significant that these percentages do not vary to any extent with the number of injections; one to two injections appear to be as efficient as three to four for the streptococci.

The results for staphylococcic cases were similar with the two types of penicillin. The percentage of cases reponding to three to four injections was 53 per cent when either type was used. The results obtained with one to two injections of crude penicillin were much different. Only 22 per cent responded to one to two injections while 67 per cent of those receiving sodium penicillin on the same dose schedule were negative after treatment. This latter figure appears inconsistent and, since only 3 cases were treated in this manner, it is discounted.

Table 3 combines the results of all treatments. The efficiency of penicillin used in streptococcic and staphylococcic cases is contrasted. Whereas 83 per cent of 47 streptococcic cases responded to treatment, only 48 per cent of 75 cases caused by *Sta. aureus* remained negative after treatment. The need for repeated administration in staphylococcic cases is emphasized when only 25 per cent of the cases which were injected less than three times responded to the treatment. Furthermore, it should be pointed out that those receiving one to two injections were treated every twenty-four hours, while those receiving three or more injections were treated every twelve hours. Therefore, the need for more frequent injections in staphylococcic mastitis is also indicated.

The data presented in table 4 show 2 typical cases of mastitis which were treated with penicillin and tested in the usual manner for six weeks afterward. Both cases show decrease in the catalase activity after treatment and disappearance of the organisms from the milk in one, 2537. This is interpreted as recovery, since all tests were between 0 and 1½ cc. of gas in the catalase test which is considered within normal limits. Although the organisms disappeared immediately, the return of the milk to normal composition was slower since healing and repair of damaged gland tissue is a relatively slow process. The other case, 1839, showed improvement but not recovery. The decrease in leucocytes as indicated by the catalase test was an indication of improvement, and the organism could not be isolated during treatment. However, after the penicillin had been eliminated from the udder, the organisms could again be recovered.

One of the outstanding characteristics of penicillin is its low toxicity to tissue. It was of interest to make observations on its effect in the udder tissue and its effect upon milk flow. Table 5 compares the average daily milk production of 9 cows for the three weeks before, during, and after treatment.

No significant lowering of milk production occurred during the week these cows were treated. All received at least four injections of 50,000 units per quarter. Rarely could any abnormality be detected upon careful physical examination of the gland or its secretion. The abnormalities which occurred were slight swellings of the gland to an occasional clot on the strip-cup. These reactions were more often seen when crude filtrates were injected than when the sodium penicillin was used.

DISCUSSION

Although penicillin is not as highly efficient as some had hoped, it has been shown to be of definite value in this disease. Elimination of the etiologic agent in 83 per cent of the streptococcic infections as reported in table 3 is satisfactory. Furthermore, penicillin causes little or no disturbance by its introduction into the udder. This is in marked contrast to some of the other agents which were used in this herd during the three years previous to penicillin therapy.

The successful treatment of 48 per cent of the *Sta. aureus* cases is not as high as desirable, which raises the question as to the reasons for the lower response in staphylococcic mastitis as compared to the streptococcic type. As shown in Pare I,[21] table 1, the sensitivity on plate tests is nearly the same for these organisms, but when bactericidal tests were made it took five to six times the concentration of penicillin to kill staphylococci as it did to kill streptococci. This difference may explain why there is a lower efficiency of penicillin in staphylococcic mastitis. It may also explain the very low efficiency of penicillin (25%) when given at 24-hour intervals as compared to 48 per cent at twelve-hour intervals. Reference to Part II,[22] tables 1 and 4, will show that when penicillin is given but once a day,

the level in the milk falls to approximately 1 to 4 units per cubic centimeter by the time, and probably for some time before, the next injection is made. If 15 to 50 units per cubic centimeter concentration is necessary for forty-eight hours to kill the staphylococci, as shown in Part I, table 3, then the penicillin level maintained in the udder with daily injections is not adequate to kill the invading Staphylococcus. Actually, Conditions are no doubt more complex in the udder than the *in vitro* tests just described, but these conditions constitute the basis for additional study. The investigation would involve the maintenance of higher levels of penicillin in the udder by even more frequent administrations than at twelve-hour intervals or by prolonging the treatment over a longer period than forty-eight to sixty hours.

Failure of penicillin therapy in five quarters infected with *Sta. aureus* was attributed to specific resistance of the organisms to penicillin. These comprised five of the resistant strains shown in Part I, table 1. In 1 case, 1977, which was infected in all four quarters with a resistant *Sta. aureus*, there was no response, for the organism was isolated without difficulty during treatment. Isolation of the organism during treatment was never successful in any other case, although quite a number were cultured during that period. Temporary improvement, such as case 1839 of table 4, usually accompanied treatment although the Staphylococcus was not permanently eliminated, but this improvement was not observed in 1977. This cow was culled from the herd not only because therapy was unsuccessful, but because the threat of spreading such a resistant strain of Staphylococcus to other cows constituted a real hazard.

It is observed in table 1 that the average dose of crude penicillin used was 38,000 units while an average dose of 204,700 units of sodium penicillin was administered, yet in table 2 there appears to be no advantage of one over the other as far as efficiency is concerned. Explanation of this might be found in referring to Part II, table 4, which shows similar penicillin levels when either 25,000 or 100,000 units are injected into the udder. There may have been undetermined factors which were in part responsible for the efficiency of crude filtrates in lower dosage, such as the concentration in the various fractions of penicillin, X, G, F, and K, in the two types of preparations used. The presence or concentration of these fractions was not determined.

The nontoxicity of penicillin in the mammary gland is reported in table 5. These results are similar to those reported by Thorp, Uhrik, and Straley;[10] Murphy and Pfau;[11] Klein, Crisman, and Moor;[8] Hardenbrook, Alberts, and Bryan;[6] and Murnane.[13]

This factor of nontoxicity is one of the most desirable from the standpoint of the dairyman who lost the milk yield for ten to fourteen days after the treatment with acriflavine or silver oxide. In many cases, the milk was being marketed before treatment, so this loss added considerably to the cost of treatment by having to take these cows out of the milking line. Complete loss of function also accompanied some treatments a few years ago but have not been described in penicillin therapy.

SUMMARY AND CONCLUSIONS

1) There is little difference in the penicillin sensitivity of streptococci and staphylococci isolated from bovine mastitis as determined by growth inhibition tests. Of 236 strains tested, only 16 grew in mediums containing 0.125 O.U. or more per cubic centimeter. *Streptococcus dysgalactiae* is slightly more sensitive than *Streptococcus uberis* or *Streptococcus agalactiae*. *Staphylococcus aureus, Str. uberis* and *Str. agalactiae* are approximately equal in sensitivity, except for 8 strains of *Sta. aureus* which were able to grow in concentrations of 1 to 10 units.

2) The bactericidal activity of penicillin does not parallel the bacteriostatic sensitivity. About 100 times the concentration required to inhibit the growth of streptococci is required to kill these same organisms. Similarly, the difference is approximately 500 times with *Sta. aureus*.

3) There are no marked differences in

the penicillin concentrations in the milk at twelve- or 24-hour intervals after the injection of doses varying between 25,000 and 200,000 units.

In instances where 25,000 to 200,000 units were administered, the interval of time between injections was of much greater importance in maintaining the level of penicillin in the udder than the amount of penicillin given. This emphasizes the need for multiple injections where high levels are required over a period of time for successful treatment.

4) Penicillin assays of foremilk averaged about 5 units higher than strippings taken at the same milking time from the same quarter.

5) A rise in temperature of 1.0 to 1.7 F. after the intramammary injection of penicillin was noted in the 3 cows observed. Only slight to negligible reactions could be detected in the udder or secretion of 53 cows treated with penicillin.

6) The response to treatment was higher (83%) in the streptococcic cases treated than the cows with *Sta. aureus* infection (48%). There was no difference in the percentage of streptococcic infections which responded to treatment with either one, two, three, or four injections. One or two injections of penicillin in staphylococcic mastitis resulted in 25 per cent success while three or more injections resulted in 50 per cent response.

7) Crude filtrates were as efficient as the sodium penicillin in the treatment of mastitis, even though the dosage of crude penicillin was about one-fifth as much as those made with the sodium salt. A possible explanation is advanced that, since the intervals of injection were approximately the same with both preparations, the timing of injections every twelve hours was of more consequence than total unitage administered.

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DDT and DDT Combined with Benzene Hexachloride for the Control and Eradication of Boöphilus Annulatus

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THE CATTLE fever tick, *Boöphilus annulatus*, has been all but eliminated from the southern United States, where it once constituted a significant hazard to cattle raising. Efforts are in progress to eradicate the ticks from the few remaining localities where they still exist. Fever ticks are, however, abundant just over our southern border in parts of Mexico where no systematic campaign to eradicate them has, as yet, been established. This situation has presented a continuous menace to the cattle industry of states adjacent to Mexico, and required in the past, inspection for ticks, of all cattle offered for importation into the United States. Between 400,000 and 800,000 head of cattle were imported into this country annually from Mexico before the outbreak of foot-and-mouth disease in that country. Since Dec. 26, 1946, the United States-Mexican border has been closed to importation of ruminants and swine from Mexico on account of the existence of foot-and-mouth disease in that country.

Cattle raisers located within the tick-infested areas of Mexico must dip their herds regularly in order to control the fever tick, otherwise their cattle would suffer severe damage from gross tick infestations. Individual cattlemen, however, cannot afford complete eradication of ticks from their herds and ranges. By so doing, the cattle would eventually lose their acquired immunity to tick fever, and become subject to the disease from surrounding sources of infestation.

The standard arsenic dip has, for many years, been the only recognized dip for the eradication of fever ticks in the United States. It has likewise been used almost exclusively for the control and destruction of fever ticks in Mexico. When properly used under suitable conditions, it has proved effective. Nevertheless, it is extremely poisonous, and severe damage has resulted in many instances from its improper use or careless handling. It provides the dipped animals with only a few days' residual protection against subsequent exposure to reinfestation, and one dipping seldom destroys all the ticks on infested animals. Therefore, cattle in infested areas must be dipped in arsenic solution at relatively frequent intervals during systematic campaigns for the eradication of fever ticks as practiced in the United States, or for merely controlling the parasites as practiced on Mexican cattle ranches. Naturally, a remedy having greater residual tickicidal qualities for the fever tick than arsenic, as well as less toxicity to man and animals, would facilitate efforts to eradicate or control the parasite wherever it exists.

The outstanding record of the more recently discovered parasiticides, namely, rotenone, DDT, and benzene hexachloride (BHC), suggested that these materials might possess the desired attributes as a treatment for the destruction of ticks. Therefore, with the approval of the Mexican government, the writer conducted in Mexico, during the spring of 1946, preliminary tests with sprays containing the newer parasiticides.[1] The sprays were tested in comparison with the standard arsenic dip on grossly infested cattle occupying tick-infested premises. The results of these preliminary tests showed that the immediate and residual tickicidal properties of suspensions containing 1 to 5 per cent of cube powder, having a 4.4 per cent rotenone content, were about equal to those of the standard arsenic dip containing 0.19 per cent of arsenious oxide. Emulsions containing 1 to 3 per cent of DDT alone, 0.5 to 1

From the Zoölogical Division, Bureau of Animal Industry, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D. C.

per cent of BHC alone, or 0.25 to 1 per cent each of the two chemicals combined, were greatly superior to arsenic solution. The sprays containing DDT possessed somewhat longer residual tickicidal properties than those containing only BHC. However, the latter chemical destroyed the fever ticks more rapidly, and sprays containing a combination of both materials gave the best results. As a result of this preliminary work, the writer was requested to return to the same area in Mexico during the fall of 1946 to conduct large-scale tests with cattle dips containing DDT alone and in combination with BHC.

After many years of utilizing arsenic dip for the control of fever ticks on their ranches and for eradication of ticks from their marketable export cattle, Mexican cattlemen have intimate knowledge of the time and labor required, the hazards involved, and the results to be expected in connection with using arsenic solutions. They were, therefore, primarily interested in determining if the new dips would produce in a shorter time equal or better results than arsenic solutions, with perhaps less effort and greater safety. They were also interested in ascertaining if, by virtue of their residual tickicidal properties, the new dips could be used to free marketable export cattle of ticks without confining the dipped animals in tick-free premises. Likewise, they wished to know if DDT dips would protect tick-free cattle against becoming infested with fever ticks while the animals were being driven through tick-infested country enroute to market or elsewhere. Ascertaining such information is not only of immediate interest to Mexican cattlemen but is also of considerable potential value to cattle raisers and livestock sanitary officials wherever fever ticks exist.

Separate tests were conducted to secure the information desired for each of the purposes mentioned and will be described separately.

MATERIALS AND METHODS

In all the dipping tests described herein, which were made prior to the outbreak of the existing epizoötic of foot-and-mouth disease in Mexico, cattle vats of the usual trench type were employed, and unheated well water was used to fill them. Commercial water-dispersible (wettable) powders that contained 50 per cent by weight of either DDT or BHC (13% *gamma* isomer content) and 50 per cent by weight of inert wetting agents were used in preparing the dips. To obtain the desired percentage, by weight, of the active ingredients in a dip, it was necessary to use twice that weight of the commercial powders. After the number of gallons of water in each vat and the weight thereof were computed, the required weight of the powders was stirred in to make the dip contain the desired percentage of actual DDT alone or actual DDT and BHC combined.

During the process of dipping, the cattle were entirely submerged in the dip but were not held there for any specific length of time. The predipping and postdipping examinations for ticks on the cattle involved in each test were conducted visually and manually while the animals were restrained in cattle chutes especially constructed for that purpose.

EXPERIMENTAL

Fever-Tick Control Test on Ranch Cattle.—This test was conducted on a large cattle ranch where fever ticks are constantly abundant. A total of 1,707 grade Hereford range cattle of all ages, including suckling calves, were used in the test. The animals harbored moderate to gross tick infestations prior to dipping and were held throughout the duration of the test in pastures known to be heavily infested with fever ticks.

The test was designed to determine if one dipping in suspensions containing a relatively low concentration of DDT alone, or a like concentration of DDT and BHC combined, would satisfactorily reduce the fever tick population on grossly infested ranch cattle and materially delay gross reinfestation on them while they were kept in tick-infested pastures.

A relatively small cattle vat was filled with 2,800 gal. of water, to which was added 1 per cent, by weight, of the commercial DDT powder, thereby making the vat contents contain 0.50 per cent of actual DDT. A total of 720 cattle were dipped in the unreplenished dip. The vat was then drained, cleaned, and refilled with water, to which was added 0.5 per cent by weight of the commercial DDT powder and a like amount of the commercial BHC powder. This dip contained 0.25 per cent of actual DDT and a like amount of actual BHC. A total of 987 cattle were dipped in the latter unreplenished dip.

In order to determine the tickicidal efficacy and stability of these two low-concentration dips, a group of 20 grossly infested animals was selected from the cattle dipped in each dip. Ten animals from each group were dipped with the first cattle that passed through each freshly prepared dip, and the remaining 10

animals in each group were dipped with the last cattle through each unreplenished dip. All these animals were marked for subsequent identification and were held, throughout the duration of the test, in a tick-infested pasture. The marked cattle were gathered and thoroughly examined for ticks at weekly intervals after the date of dipping.

On those animals that passed through the freshly prepared DDT dip, only a few engorged and unengorged adult female ticks were found alive on the seventh and fourteenth days. These few surviving ticks were not present on the twenty-first day. At this time, however, a few newly acquired larvae and a few nymphs were found on the test animals. This indicated that, following the fourteenth day after dipping, a few newly attached larvae survived on the hosts and that some of the early survivors had developed into nymphs. By the twenty-eighth day, some of the nymphs had developed into young adults, and thereafter a few ticks in all stages of development could be found on the cattle. However, none of the animals in this test lot became more than lightly reinfested with fever ticks during a postdipping period of seven weeks, at which time the tests were concluded. On the animals that passed through the freshly prepared dip containing both DDT and BHC, no living ticks were found on the seventh and fourteenth days. Thereafter, however, the occurrence, development, and degree of reinfestation on this test lot of cattle were almost identical with those observed on the cattle dipped in the freshly prepared DDT dip.

The dead ticks on the dipped cattle soon lost their natural appearance. They became flattened and dark in color and eventually became desiccated and were gradually dislodged from the animals. These processes occurred somewhat more slowly on the cattle that were dipped in the DDT dip than on those that passed through the combined DDT-BHC suspension.

The tickicidal properties of both dips apparently became somewhat less effective as dipping progressed. Although the used DDT dip destroyed most of the ticks on the cattle that passed through it, some ticks in all stages of development continued to survive on the animals. The used DDT-BHC dip was slightly more efficient. It killed nearly all the ticks on the cattle that were dipped in it, but some young and mature adults survived on the animals. Since it was apparent that the used, unreplenished dips had not destroyed all the ticks on these two lots of cattle, further postdipping examinations of them were discontinued after the fourteenth day.

Fever-tick Eradication Test with Cattle Confined in Tick-free Premises Subsequent to Dipping.—This test was conducted in conjunction with the test already described. It was designed to determine if one dipping in the same low-concentration dips would eradicate fever-tick infestations from grossly infested cattle if the animals were kept in tick-free premises subsequent to dipping.

A tick-free pen was prepared by thoroughly burning the entire ground surface with weed burners. The pen was located adjacent to the ranch dipping corrals and was entirely inclosed by a tightly fenced, 10-foot wide alleyway. It contained feeding and watering facilities and an inspection chute.

Twenty grossly tick-infested, grade Hereford cows were selected for the test. They were divided into four lots of 5 cattle each and were marked for identification. As in the previously described tick control test, one lot was dipped in the freshly prepared dip that contained 0.5 per cent of actual DDT, and a second lot in the same used, unreplenished dip. The third lot was dipped in the freshly prepared dip that contained 0.25 per cent each of DDT and BHC and the fourth lot in the same used dip. Immediately after dipping, the animals were put into the tick-free pen where they remained until the conclusion of the test. The animals were examined for ticks on the first, second, third, fifth, and seventh days after dipping, and at weekly intervals thereafter.

The freshly prepared DDT dip required more than twenty-four hours to kill the ticks on the cattle that were dipped in it, as no dead ticks were found on the animals during the first examination. On the second day, however, all ticks except some young and mature adults were dead. Thereafter, the surviving ticks became fewer in number, and on the seventh and fourteenth days, only very few engorged and unengorged adult females remained alive on the animals, and none was found on the twenty-first day. On subsequent examinations, which continued for an additional four weeks, the cattle in this lot remained free of living ticks.

The freshly prepared DDT-BHC dip killed the ticks more rapidly on the cattle that passed through it. On the first day after dipping, all ticks were dead, except a few moribund engorged and unengorged adult females. These had all perished by the fifth day and no living ticks were found on this lot of cattle during the subsequent six weeks.

Both of the used, unreplenished dips were less effective. Although the used DDT dip destroyed most of the ticks on the cattle dipped in it, some ticks in all stages of development, except larvae, survived on the animals. The surviving ticks appeared to develop normally, as no nymphs remained on the cattle on the seventh and fourteenth days, but young adults

were then more numerous. The used DDT-BHC dip was somewhat more effective. Only a few moribund, adult female ticks and some nonmotile nymphs showed evidence of vitality during the first three days after dipping. Thereafter, the moribund adults died but some of the nonmotile nymphs apparently survived and molted, as a few young adults were found on this test lot of cattle on the seventh day, and few mature adults had developed by the fourteenth day. Since both lots of cattle that passed through the used, unreplenished dips continued to harbor living ticks on the fourteenth day after dipping, they were removed from the tick-free pen and that phase of the test was concluded.

Fever Tick Eradication Tests with Marketable Export Cattle on Tick-infested Pastures.—The findings observed in the two tests described prompted the inauguration of these tests. The tests involved six different lots of marketable cattle totaling 1,901 animals that were destined for export to the United States. Each lot was due for export on a definite date. Therefore, the owners were anxious to determine if dips containing relatively large concentrations of DDT alone, or similar concentrations of DDT and BHC combined, would quickly and safely rid the cattle of fever-tick infestations, and if this could be accomplished without confining the animals in tick-free premises. Therefore, in order to be certain that the initial and residual tickicidal qualities of the dips would be maintained throughout, the owners used excessive amounts of DDT and BHC in charging and replenishing their cattle vats and, in most instances, resorted to excessive dippings of the cattle.

At one ranch, a medium-sized vat was filled with 3,600 gal. of water, and a sufficient amount of the commercial DDT powder was added to make the dip contain 1 per cent of actual DDT. A herd of 470 tick-infested, grade Hereford steers was then dipped. Ten grossly infested animals were selected from the herd, marked for identification, and placed in a nearby tick-infested pasture. The herd was returned to a tick-infested range. The 10 marked cattle were examined on the second, seventh, and eleventh days after dipping. A few mature adult female ticks continued to survive on the animals, so the entire herd was redipped on the eleventh day of examination. Before the cattle were dipped the second time, the vat was replenished. Although only 1/10 of the original charge of water was required to refill the vat, the owner added commercial DDT powder in an amount equal to about 1/16 of that originally used in charging the vat. The 10 marked animals were again examined on the fifth day after the second dipping. One mature, living tick was found, so the entire herd was dipped a third time on that date. Prior to the third dipping, a sufficient quantity of the commercial BHC powder was added to the vat contents to make it contain 0.5 per cent of actual BHC in addition to the DDT therein. The marked animals and several unmarked steers from the herd were examined on the fourth day after the third dipping. No living ticks were found, and very few dead ticks remained on the cattle. The entire herd was subsequently shipped to the United States border where, on the tenth day after the third dipping, each animal was thoroughly examined by government inspectors, assisted by the writer. No living ticks were found, and the entire herd, in compliance with Government regulations, was dipped once in arsenic solution and exported to the United States.

At the same ranch, two additional lots of tick-infested, grade Hereford cattle, one consisting of 26 steers and another of 130 aged bulls, were dipped in the used DDT-BHC dip. The steers were dipped only once and the bulls twice, five days apart. All 26 steers and 11 grossly infested animals from the bull herd were held in the tick-infested pasture while the remainder of the bull herd was returned to a tick-infested range. After five days had elapsed following the first dipping, no live ticks were found on any of the pasture-held steers or bulls. Nevertheless, the entire bull herd was redipped at that time in the unreplenished dip. On the sixteenth day after the date of the first dipping, both lots of cattle were presented for export inspection at the U. S. border. No living ticks were found on any of the once-dipped steers or on the twice-dipped bulls, and only a few dead ticks remained on the cattle.

Two additional lots of grade Hereford steers, one consisting of 5 grossly infested animals and the other 370 steers harboring moderate to heavy tick infestations, were dipped at the same ranch in the same unreplenished DDT-BHC dip. The lot of 5 steers was subjected to a double dipping, which consists of redipping cattle before they become completely dry following their first dipping. These animals, and 10 heavily infested steers from the second lot, were marked for identification and held in the tick-infested pasture, while the remaining animals in the second lot were returned to the tick-infested range.

On the second day after dipping, a few moribund adult female ticks were found on the marked steers, but on the twelfth day no living ticks were found and but few dead ticks remained on the animals. Nevertheless, on that day, all 370 steers comprising the second lot were dipped a second time. For this dipping, the vat was replenished, and while only 1/6 of the original charge of water was required, the owner added BHC powder in an amount equal to ¼ the original charge of that

material. Three days after the date of this second dipping, the 370 twice-dipped steers and the lot of 5 once double-dipped steers were presented for export examination at the U. S. border. No live ticks were found and the animals were duly exported.

At another ranch, a large vat was filled with 5,400 gal. of water, and sufficient quantities of commercial DDT powder and commercial BHC powder were added to make the dip contain 1 per cent of actual DDT and 0.33 per cent of actual BHC. A herd of grade Hereford cattle composed of 600 cows and 300 calves was then doubled-dipped. Six grossly infested cows were selected from the herd and held in a nearby tick-infested pasture, and the remainder of the herd was returned to a tick-infested range. The six pasture-held cattle were examined on the third, sixth, and eleventh days after dipping. A few moribund adult female ticks were found on the third and sixth days, but no live ticks were found thereafter. Nevertheless, the entire herd was again double-dipped on the eleventh day after the first dipping.

Before beginning the second dipping, the vat was replenished with water and excessive quantities of DDT and BHC. Only about ¼ of the amount of water used to charge the vat originally was required to refill it, but over ½ the original amount of commercial DDT powder and slightly less than the total original amount of commercial BHC powder used were added. These excessive amounts of DDT and BHC were used by the owner in order to be certain that his export cattle harbored no living ticks, and to determine if such high concentrations of the insecticides in a dip would be injurious to cattle.

Following the second dipping, the herd was driven overland through tick-infested country for a distance of about 65 mi. to feeding corrals near the United States border. Upon their arrival at the corrals, five days later, they were double-dipped a third time in a large vat filled with 7,600 gal. of water, to which sufficient quantities of commercial DDT powder and commercial BHC powder had been added to make the dip contain 0.5 per cent of actual DDT and 0.33 per cent of actual BHC. On the third day after this dipping, the herd was presented for export inspection at the United States border. No living ticks and very few dead ticks were found on the animals; they were passed for export to the United States. At no time did the cows and calves comprising this herd evidence any injurious effects as a result of the excessive double dippings to which they were subjected.

Protective Dipping Test Involving Tick-free Cattle.—A herd of 1,100 grade Hereford cows, originating on a tick-free ranch, and consequently not immune to piroplasmosis, was dipped once at the home ranch in freshly prepared dip containing 0.66 per cent of actual DDT. Immediately thereafter, the herd began an overland drive of approximately 70 mi., the journey requiring five days, most of which was spent in grossly tick-infested brush country. Upon arriving at a Mexican railway point of shipment on the fifth day, the herd was again dipped in freshly prepared dip containing 0.66 per cent of actual DDT. On the following day, the herd was shipped to the United States border and two days later was presented for export examination. No living ticks were found, and the herd was duly exported and shipped to a ranch in south-central Texas. Some sixty days later, the owner reported that none of the animals in the herd had shown symptoms of piroplasmosis.

DISCUSSIONS

The results obtained and the observations made in connection with the tests described in this paper show that aqueous suspensions of DDT alone or in combination with BHC are effective and safe for the destruction of cattle fever ticks. The suspensions not only exterminated gross tick infestations on dipped cattle but, through their residual properties, materially retarded normal reinfestation when the animals were either held in heavily tick-infested pastures or driven through such areas. The inclusion of BHC in the suspensions resulted in a quicker kill of all ticks on infested cattle than occurred when DDT was used alone.

Repeated dippings of cattle, of all ages, in suspensions containing relatively high concentrations of both chemicals resulted in no discernible discomfort or injury to the animals, and strenuously exercising them at any time after dipping likewise produced no untoward results.

The commercial, water-dispersible, or so-called wettable, powders containing DDT or BHC mixed readily with the water in the dipping vats but, when the vat contents were not being agitated, the products settled to the bottom quite rapidly. Therefore, following cessations in the dipping procedure, the vat contents required vigorous stirring before dipping was resumed.

The dips containing relatively low concentrations of DDT alone, or DDT combined with BHC, gradually lost some of their tickicidal properties as dipping progressed.

This was to be expected, since neither chemical is soluble in water but is merely suspended there in the form of fine particles. Therefore, it is reasonable to assume that the active ingredients in the dips were screened out in the hair of the dipped cattle more rapidly than was the water.

The ticks destroyed by the dips soon became withered and dark in color. Most of them remained firmly attached to the skin of the dipped animals, and it was usually a few days before any appreciable number of them became dislodged. A few were found on some cattle thirty-five days after the date of dipping.

The dipping tests were of insufficient duration to determine the tickicidal stability of the DDT or DDT-BHC dips that remained in vats for any length of time. However, the owner of the ranch vat in which the 900 export cows and calves were twice double-dipped in dip containing excessive quantities of DDT and BHC has reported that the old dip, which had been considerably diluted by rain water, was still effective against fever ticks after standing in the vat for seven months.

As previously stated in this paper, the standard arsenic dip has for many years been considered the remedy of choice for destroying cattle fever ticks and is still the only dip officially recognized for the eradication of these pests in the United States. Therefore, a brief summary of the comparative advantages and disadvantages of arsenic dip with those of DDT and DDT-BHC dips is considered appropriate.

1) Tickicidal Action—One dipping in DDT or DDT-BHC dips, especially the latter, produces a more rapid and complete destruction of fever ticks than does one dipping in the standard arsenic dip.

2) Residual Qualities—The DDT and DDT-BHC dips apparently remain toxic to all newly attaching larval ticks on dipped cattle for about two weeks and thereafter continue to possess some tickicidal action, preventing gross reinfestation for an additional period of at least five weeks. Residual effects of the standard arsenic dip apparently last only a few days.

3) Required Frequency of Application.—In campaigns for the eradication of fever ticks in infested areas, as practiced in the United States, it is necessary to use arsenic dip every two weeks in order to destroy newly attached ticks before they become mature and leave the host animals. By virtue of their residual qualities, the DDT and DDT-BHC dips, especially the latter, could in all probability be used half as often to secure the same results. Likewise, for adequately controlling the tick population on cattle in infested areas, as practiced on Mexican ranges, arsenic dip must be used every three to four weeks, whereas the use of DDT or DDT-BHC dips every seven to eight weeks would probably suffice for this purpose.

4) Safety Factors.—Arsenic solutions are highly poisonous to both man and animals, and the range of concentration where they become noneffective against fever ticks and where they produce severe damage or death to animals dipped in them is quite limited. On the other hand, DDT and DDT-BHC dips possess no such dangerous qualities and were used safely and effectively in relatively wide variations of concentration. Whereas considerable restriction must be exercised in the handling of animals following dipping in arsenic solution, no such restrictions were necessary when the DDT and DDT-BHC dips were used.

5) Cost Factor.—At current prices, the dips containing the lowest effective concentrations of DDT or DDT-BHC cost about twice as much as the standard arsenic dip. However, depending upon relevant circumstances, the actual cost of the dip material may become insignificant when factors such as safety, rapid and lasting efficacy, and the freedom of handling dipped animals, as afforded by the use of DDT or DDT-BHC dips, are considered.

6) Stability Factors.—The stability of used arsenic dip is well known. It has been used effectively time after time when properly replenished and kept relatively free of extraneous material. No such established information is, as yet, available for the used DDT and DDT-BHC dips. It is known that the latter dips lose their tickicidal efficacy through use somewhat more rapidly than arsenic dips and require more frequent replenishment. Upon standing, some gradual separation of the water and active ingredient in arsenic solutions occurs. This, however, is readily corrected by ordinary stirring. On the other hand, the active ingredients in DDT or DDT-BHC suspensions settle out quite rapidly upon standing, requiring more vigorous stirring before being used.

7) Ascertaining Strength of Used Dip.—There is a reliable field test for determining the actual concentration of arsenious oxide in freshly prepared or used arsenic dip. The safe and effective use of the dip requires such

a test outfit. No such field test has, as yet, been developed for use with DDT or DDT-BHC dips, and their tickicidal efficacy throughout prolonged usage can be maintained only by frequent and adequate additions of the active ingredients. However, no danger appears to be involved when excessive quantities of the ingredients are added to the dips.

SUMMARY AND CONCLUSIONS

1) Cattle dips containing various concentrations of DDT, and DDT combined with BHC, in aqueous suspensions, were tested in four separate field trials to determine the efficacy of the dips for: (a) controlling fever ticks on range cattle, a necessary practice on tick-infested Mexican ranches; (b) destroying all existing ticks on infested cattle that were confined in tick-free premises after dipping; (c) accomplishing this same tick-cleansing purpose without utilizing tick-free premises; and (d) protecting tick-free cattle against becoming infested with fever ticks during drives through tick-infested areas.

2) Approximately 1,700 tick-infested, grade Hereford range cattle of all ages were used to evaluate the efficacy of the dips for the first purpose enumerated above. A group of 20 such animals was used in the second test. Six lots of similar cattle, totalling approximately 1,900 animals, were involved in the third test, and a herd of 1,100 tick-free grade Hereford range cattle was used in the fourth test.

3) One dipping in freshly prepared dips containing only 0.5 per cent of DDT or 0.25 per cent each of DDT and BHC resulted in the complete elimination of all the existing tick infestation on dipped cattle. It also protected the constantly exposed cattle from becoming reinfested with larval ticks for about two weeks, and thereafter materially inhibited the normal extent of fever tick infestation for an additional period of at least five weeks. Thus, it is evident that the use of such dips at relatively infrequent intervals of perhaps seven to eight weeks would adequately control the tick population on continuously exposed ranch cattle.

4) One dipping in freshly prepared dip containing only 0.5 per cent of DDT killed all ticks except a very few engorged and unengorged adult females within five days, and the few surviving ticks were not present on the twenty-first day. All ticks on similarly infested cattle were destroyed in five days by a single dipping in dip that contained only 0.25 per cent each of DDT and BHC. Therefore, these dips proved adequate for eradicating all existing ticks from cattle that were confined in tick-free premises subsequent to dipping.

5) Two dippings, eleven days apart, in freshly prepared dip containing 1 per cent of DDT, failed to destroy immediately all the existing ticks on dipped cattle. As in the preceding test, a few mature adult female ticks survived the dip containing DDT alone. Such dips cannot, therefore, be relied upon to eliminate quickly the fever tick infestation on cattle being prepared for export shipment.

6) The addition of BHC to DDT dips resulted in a comparatively rapid and complete tick kill. One dipping in dips containing 1 per cent of DDT combined with 0.33 per cent to 0.5 per cent of BHC destroyed all existing ticks on grossly infested cattle in about five days and prevented their becoming reinfested during eleven- to sixteen-day periods of exposure in tick-infested pastures. One or two additional dippings in such dips at five- to twelve-day intervals produced no injurious effects to the cattle. Such dips, therefore, may be safely and effectively used to cleanse grossly tick-infested cattle without resorting to the use of tick-free premises during the process.

7) Tick-free cattle that were susceptible to piroplasmosis were dipped once in dip containing 0.66 per cent of DDT alone just prior to leaving their tick-free range, and again in like dip upon completion of a five-day drive through heavily tick-infested country. The animals were apparently protected against the exposure to fever ticks and tick-borne piroplasmosis, as no symptoms of the disease occurred later among the animals.

References

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The Potentiating Action of Sulfonamides on the Brucella Antibody-Complement System

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AN INVESTIGATION of the potentiating action of sulfonamides on the Brucella antibody-complement system was instigated after it was repeatedly demonstrated by the writer that the precipitation of sodium sulfadiazine in undiluted blood serum, by means of a dilute solution of phosphoric acid, removed most of the *gamma* globulin fraction with the precipitate. In other words, this serum fraction united with the sulfadiazine as it came out of solution. Its presence was proved by an electrophoretic analysis of the precipitate after it had been dissolved in 0.1N NaOH and dialyzed to remove sodium sulfadiazine.

TABLE I—The effect of Six Sulfonamide Compounds on the Growth of Brucella Abortus

Drug used	Period of inc. (hr.)	Dilution at which no visible growth occurred*	
		Without serum	With serum**
Na-sulfanilamide	24	1:1.6 x 10^4	1:6.4 x 10^4
	72	<1:1 x 10^3	1:3.2 x 10^4
Na-sulfapyridine	24	1:1.6 x 10^5	1:3.2 x 10^5
	72	<1:1 x 10^3	1:1.6 x 10^5
Na-sulfathiazole	24	1:1.6 x 10^5	1:1.28 x 10^6
	72	<1:1 x 10^3	1:1.28 x 10^6
Na-sulfamethazine	24	1:8 x 10^4	1:2.56 x 10^6
	72	<1:1 x 10^3	1:3.2 x 10^5
Na-sulfamerazine	24	1:8 x 10^3	1:2.56 x 10^6
	72	<1:1 x 10^3	1:3.2 x 10^5
Na-sulfadiazine	24	1:1.6 x 10^5	1:2.56 x 10^6
	72	<1:1 x 10^3	1:6.4 x 10^5

*10^6 *Br. abortus* added to each dilution.
**0.5 cc. fresh, normal rabbit serum added to each dilution. Inoculated control tube of medium containing 0.5 cc. of the fresh, normal rabbit serum showed no inhibition of growth.

Electrophoretic patterns obtained by the Longsworth[1] scanning method in the Tiselius apparatus on the serum before treatment, and on the protein recovered from the sulfadiazine precipitate, showed that approximately 90 per cent of the *gamma* globulin fraction and 10 per cent of the albumin fraction were removed from the serum in the precipitate. It was also shown that a solution of this fraction possessed marked bacteriostatic and bactericidal activity for *Brucella abortus* in the presence of serum complement.[2]

Several workers have already demonstrated in *in vitro* experiments that the sulfonamides increase, by some means, the action of certain defensive mechanisms of the host. An increased phagocytic activity of phagocytic cells has been noted.[3] Finkelstein and Birkeland[4] advanced the opinion from their studies that sulfanilamide or a serum-sulfanilamide complex acted as an opsonin. Welch[5] noted that the blood of Brucella-infected guinea pigs and human beings showed a high degree of opsonic activity when sulfanilamide was present and advanced the hypothesis that the drug acted as a neutralizing agent on endotoxin or some aggressin-like substance which was present in the blood and prevented phagocytosis. Osgood[6] had previously advanced a similar hypothesis, but his pertained to the neutralization of bacterial toxins by the drug.

In a series of previous *in vitro* experiments,[7] designed to determine the mechanism of immunity to and recovery from brucellosis, it was shown that undiluted, fresh serum or plasma from most normal as well as immune cattle contains specific antibodies which in combination with complement will kill large numbers of Brucella organisms (*abortus, suis, melitensis*). A similar effect could not be demonstrated in undiluted, fresh serum or plasma from Brucella-infected cattle.

In more recent studies, the results of

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which will be presented in this paper, it has been demonstrated that the blood serums of Brucella-infected animals, such as cattle, guinea pigs, and rabbits, contain antibodies which, in the presence of complement, will delay the growth of Brucella organisms *in vitro*. This property in serums can be demonstrated only in adequate dilutions in the presence of complement. In other words, the growth-inhibiting action occurs in a zone beyond a certain dilution of the serum. This anomalous phenomenon was first observed and described by Neisser and Wechsberg in 1910.[8]

TABLE 2—Effect of Sulfadiazine on Brucella Suis Infection in Guinea Pigs*

Experiment No.	Amt. NaSD fed/pig†	Days between exposure and feeding	No. treated / No. controls	No. treated, infected / No. controls, infected
1	2 Gm. daily, 7 days	7	10 / 10	0 / 10
2	1 Gm. daily, 7 days	7	10 / 10	0 / 10
3	0.25 Gm. daily, 7 days**	7	22 / 10	0 / 10
4	1 Gm. daily, 7 days	27	10 / 10	9 / 10
5	0.25 Gm. daily, 7 days	28	10 / 10	9 / 10

*Infecting dose of organisms, 1,000 cells.
**Average conc. of sulfadiazine in blood, 29 mg./100 cc.
†Drug mixed with 200 Gm. of dry feed.

A similar phenomenon of this nature was first described by Pfeiffer[9] in *in vivo* experiments in which immune serum was used in the treatment of infections. Large doses of a given immune serum were found to have no more therapeutic effect against infection than small amounts. This effect has come to be known as a prozone phenomenon.

Experiments conducted in this laboratory have demonstrated that a dilution as high as 1 : 160,000 of the soluble sodium salts of several of the sulfonamides in a suitable liquid culture medium will delay or retard the multiplication of Brucella for twenty-four to thirty-six hours. After this period of incubation, visible growth appears in dilutions of the same drugs as low as 1 : 1,000.

If the results of *in vitro* experiments conducted in this laboratory and elsewhere are an indication of the therapeutic value of the sulfonamide compounds, one would expect to obtain as favorable results from their use in the treatment of Brucella infections as have been obtained in many other diseases. The results obtained by Chinn[10] and Menefee and Paston[11] from the treatment of guinea pigs with sulfanilamide shortly after experimental infection with *Brucella abortus* indicated that this agent had possibilities as a therapeutic agent for the treatment of naturally occurring brucellosis. In recent years, experiences of physicians and veterinarians who have used sulfonamides in the treatment of human and animal brucellosis have failed to confirm the experimental findings.

TABLE 3—Comparison of Bacterial Growth Inhibiting Action of Serums from Brucella Suis—Infected Guinea Pigs

Days infected when bled	Inc. period (hr.)	Degree of growth, turbidity[a] Dilutions of serum, 1: 1×10^1 to 4×10^1	8×10^1	1.6×10^2	3.2×10^2 to 2×10^3	4×10^3	Bacteria control 5 cc. of culture medium
7	24	—	—	1+	2+	2+	2+[b]
	72	6+	6+	6+	6+	6+	6+
30	24	2+	1+	1+	—	1+	2+[b]
	72	6+	6+	6+	6+	6+	6+

[a] 10^5 *Br. abortus* added to each dilution and to controls.
[b] Control tube of medium also contains 0.2 cc. of fresh, normal rabbit serum. An additional control tube of medium containing only added organisms not indicated in this and other tables showed 2+ growth at end of twenty-hour hours and 6+ at end of seventy-two hours.
0.2 cc. fresh, normal rabbit serum added to each dilution.
— = no visible growth. + = degree of growth.

During the period of study of the action of the sulfonamide compounds, it was demonstrated that, when fresh serum from a normal rabbit or cow was added to dilutions of the soluble sodium salts in a suitable liquid culture medium containing live Brucella organisms, the temporary growth-inhibiting effect of the drugs was enhanced to a bactericidal effect. Furthermore, this enhancement could be demonstrated in dilu-

tions of the drugs far beyond their temporary growth-inhibiting action when used alone. Strange as it may seem, no similar action could be demonstrated when fresh serum from infected animals was used in place of normal serum. The inhibiting action of the drugs was actually decreased.

Extensive and laborious experiments have been conducted in the hope that an explanation might be found for the enhanced action of the sulfonamides in the presence of fresh, normal serum and their diminished activity in the presence of fresh serum from infected animals. The drug selected for study in these experiments was sulfadiazine and its soluble sodium salt.

The following experimental data show that the failure of sulfadiazine to inhibit the growth of Brucella organisms in undiluted or diluted blood serum from infected animals is due to the prozone antibody phenomenon and that, when it is prevented from occurring, the blood serum will become highly bactericidal *in vitro* and *in vivo*.

METHODS

Sulfonamide Preparations.—The *in vitro* experiments were made with the sodium salt of sulfadiazine and the *in vivo* ones with sulfadiazine. A few comparative *in vitro* experiments were also made with the sodium salts of sulfanilamide, sulfapyridine, sulfathiazole, sulfamerizine, and sulfathiazine. Solutions of the salts were prepared in distilled water on the day they were used and sterilized by filtering through a D6 Hormann filter pad in a Seitz filter.

Preparation of Blood Serum.—Blood was collected aseptically from the animals under study and permitted to clot at room temperature. If the complement of the serum sample was utilized instead of added serum complement, the bacteriostatic or bactericidal determinations were made within four hours after collection. Fresh rabbit serum was the source of added complement. This was collected aseptically and used within four hours after the serum had begun to separate from the clot. No serum was used as a source of complement if a previous titration showed inhibition of growth of *Br. abortus* in a dilution of 1 : 5.

Bacterial Suspension.—A smooth strain of *Br. abortus* was used in all *in vitro* titration experiments unless otherwise stated. The organisms were grown on beef liver infusion agar slants for twenty-four hours at 37 C. The growth was removed and suspended in sterile dilution fluid. The suspension was standardized to a live colony count of 1×10^9 per cubic centimeter, and 0.1 cc. of this suspension was added to each tube in all titration experiments. The bacterial suspensions of the Brucella species used for inoculating guinea pigs were prepared in the same way.

Titration of Activity of Sulfonamides and Antibody-Complement Mixtures. — All titration experiments, regardless of whether they pertained to the determination of the activity of the sulfonamides, the antibody-complement system, or a combination of the two, were made in a liquid culture medium containing 1.5 Gm. of tryptose peptone, 0.5 Gm. glucose, 0.5 Gm. sodium chloride, and 0.5 Gm. thiamin

TABLE 4—In Vitro and In Vivo Potentiating Effect of Sulfadiazine on the Growth Inhibiting Action of Serums from Brucella Suis-Infected Guinea Pigs (Infected for Seven Days)

Guinea pig No.	Amt. of agents added to serum dil.	Inc. period (hr.)	Degree of growth, turbidity[a] Dilutions of serum, 1: 1×10^1 to 8×10^1	1.6×10^2	3.2×10^2	6.4×10^2	1.28×10^3	2.56×10^3	5.12×10^3	Bacterial control 5 cc. of culture medium
1	0.2 cc. NRS	24	—	1+	2+	2+	2+	2+	2+	2+[c]
		72	6+	→	→	→	→	→	→	6+
2	0.2 cc. NRS 0.02 Mg. NaSD	24	—	—	—	1+	1+	1+	2+	2+[d]
		72	—	—	1+	4+	4+	4+	4+	4+
2[b]	0.2 cc. NRS	24	—	—	—	—	—	—	1+	2+[c]
		72	—	1+	1+	2+	6+	6+	6+	6+

[a] 10^6 *Br. abortus* added to each dilution.
[b] Fed ad lib. 0.25 Gm. sulfadiazine over 24-hour period before collecting blood sample.
[c] Control tube of medium also contains 0.2 cc. of NRS.
[d] Control tube of medium also contains 0.2 cc. of NRS and 0.02 mg. of NaSD.
NRS = Fresh, normal rabbit serum. NaSD = Sodium sulfadiazine.
— = no visible growth. + = degree of growth.

hydrochloride in 100 cc. of distilled water. The pH was adjusted to 6.7 with H_3PO_4. It was then filtered through filter paper to clarify, distributed in 5- or 10-cc. amounts into sterile test tubes, and sterilized at 115 C. for fifteen minutes. When this medium is inoculated with 1×10^5 *Br. abortus* organisms, visible growth can be seen after twenty-four hours of incubation at 37 C.

The maximum growth-inhibiting or bactericidal action of the drugs or of the serum-containing antibody was determined by making serial, twofold dilutions of the agent in 5 cc. of the culture medium. The other agents, such as serum-complement and organisms, were added to each tube in constant amounts; the organisms were always added last. The tubes were shaken and placed in a 37 C. room. The period of incubation, as a rule, extended to seventy-two hours. The degree of growth was measured (visible turbidity) at intervals of twenty-four hours and indicated by using – or + signs.

EXPERIMENTS AND RESULTS

The Effect of Six Sulfonamides on the Growth of Brucella Abortus.—In order to determine whether several of the more widely used sulfonamides retarded or prevented the growth of *Br. abortus*, sterile sodium salts were diluted progressively from 1 : 1×10^3 to 1: 2×10^7 in the fortified tryptose medium inoculated with *Br. abortus*.

The data in table 1 are representative of the results of repeated tests made to determine the bacteriostatic and bactericidal activity of the sodium salts of six sulfonamide compounds on the growth of *Br. abortus* in the fortified tryptose medium. Duplicate sets of the same dilutions of the compounds were made, and to one set was added 0.5 cc. of fresh, normal rabbit serum. This serum contained, in addition to complement, a small amount of natural Brucella antibody.

Each of the compounds without serum showed its maximum growth-retarding effect (absence of turbidity) during the first twenty-four hours. Sodium sulfanilamide showed the least action of all. Visible growth appeared in all dilutions of all the compounds by the forty-eighth hour of incubation.

The presence of 0.5 cc. of fresh rabbit serum in each dilution of the compounds caused or brought about a remarkable bactericidal activity of the drugs or the added antibody-complement system. In those dilutions of the drugs containing fresh serum, in which no visible growth appeared in seventy-two hours, one was able to demonstrate the absence of live organisms. Many other comparable experiments, which were made during the course of this study, have shown conclusively that the increased action of the drugs in the presence of a sufficient amount of fresh serum could be attributed to the activity of the antibody-complement system. For example, by adding a sufficient amount of specific anti-

TABLE 5—In Vitro and In Vivo Potentiating Effect of Sulfadiazine on the Growth Inhibiting Action of Serums from Brucella Suis—Infected Guinea Pigs (Infected for Thirty-Three Days)

Guinea pig No.	Amt. of agents added to serum dil.	Inc. period (hr.)	Degree of growth, turbidity[a] Dilutions of serum, 1: 1×10^1	2×10^1	4×10^1	8×10^1	1.6×10^2	3.2×10^2	6.4×10^2	1.28×10^3	2.56×10^3 to 2×10^4	4×10^4	Bacterial control 5 cc. of culture medium
1	0.2 cc. NRS	24	2+	2+	1+	1+	—	→					2+[d]
		72	6+	→									6+
1	0.2 cc. NRS 0.02 mg. NaSD	24	1+	—	→								1+[e]
		72	6+	4+	4+	4+	—	→					4+
1	0.5 cc. NRS 0.02 mg. NaSD	24	—	→									1+[e]
		72	—	→									4+
2[b]	0.2 cc. NRS	24	2+	2+	2+	2+	1+	1+	—	—	—	—	2+[d]
		72	6+	→									6+
3[b c]	0.2 cc. NRS	24	—	→									2+[d]
		72	—	—	—	—	—	—	—	3+	4+	6+	6+

[a] 10^5 *Br. abortus* added to each dilution. — = no visible growth. + = degree of growth.
[b] Fed *ad lib.* 0.25 Gm. sulfadiazine over 24-hour period daily for three days. Blood sample collected end of third day.
[c] Thirtieth day of infection injected intraperitoneally with 2 cc. of normal rabbit serum.
[d] Control tube of medium also contains 0.2 cc. NRS.
[e] Control tube of medium also contains 0.02 mg. NaSD.
NRS = Fresh, normal rabbit serum. NaSD = Sodium sulfadiazine.

body and complement to each of the dilutions of sodium sulfadiazine, bactericidal action was observed in a dilution of 1 : 1 x 10^7. A similar action has not been obtained in the presence of heated serum or a serum devoid of complement.

Effect of Sulfadiazine on Brucella Suis Infection in Guinea Pigs.—In table 2 are shown the results of a series of experiments designed to show the possible therapeutic value of sulfadiazine on the course of *Brucella suis* infection in guinea pigs. *Brucella suis* was used as the test organism in these experiments, principally because the injection of a small number of virulent cells will produce extensive changes in the organs of the guinea pig in less than thirty days.

Several groups of 10 pigs each were inoculated subcutaneously with 1 x 10^8 live and virulent *Br. suis* cells. Administration of the drug was begun after the pigs had been infected for seven or for nearly thirty days. Sulfadiazine was mixed with a sufficient amount of finely ground grain so that the mixture would last each group of 10 pigs for a twenty-four hour period of feeding; the mixture was replenished each day for seven days. The amount of the drug that was available for each pig each day is indicated in table 2. The concentration of the free drug in the blood of those that received 1 and 2 Gm./day was found to average 30 mg./100 cc. of blood, and 4 to 25 mg./100 cc. of the blood of those receiving 0.25 Gm./day.

The guinea pigs in each group were killed twenty days after the seventh day of treatment and the tissues cultured for the presence of *Br. suis* organisms. The failure to culture organisms from the tissues was used as the criterion of recovery from infection.

All of the guinea pigs which were infected for seven days when the feeding of the drug was begun were found free from infection on autopsy. All controls, however, were found infected. The daily feeding of the drug for seven days after the guinea pigs had been infected for twenty-seven and twenty-eight days failed to affect the course of the disease in 18 of the 20 pigs used. On autopsy, all of the 18 showed extensive gross changes in the organs, characteristic of infection, and also positive cultures.

It would be natural to attribute the curative effect of the drug, when administered early in infection, to its direct action on the small number of organisms present in the tissues and its failure to cure, when administered after generalized infection had occurred, to the extent to which the disease had progressed. Such a premise would seem logical if it could be shown that the specific antibody, which appears in the blood serum shortly after infection occurs, does not influence in any way the activity of the drug *in vivo*. The results of the experiments which follow will show the rôle played by the antibody-complement system in the *in vitro* and *in vivo* action of sulfadiazine on the growth of Brucella organisms.

Activity of Specific Antibody in Serums from Brucella Suis-Infected Guinea Pigs.—The results set forth in table 3 are typical examples of the manner in which specific serum antibody of guinea pigs or other ani-

TABLE 6—The potentiating Effect of Na-Sulfadiazine and Normal Rabbit Serum on the Bacterial Growth Inhibiting Action of Serum from a Brucella Abortus—Infected Cow

		Degree of growth, turbidity[a] Dilutions of serum, 1:									
Amt. of agents added to each serum dilution	Inc. period (hr.)	1 x 10^3 to 1.6 x 10^4	3.2 x 10^4	6.4 x 10^4	1.28 x 10^5	2.56 x 10^5	5.12 x 10^5	1 x 10^6	2 x 10^6	4 x 10^6	Bacterial control 5 cc. of culture medium
0.5 cc. NRS	24	2+	1+	—	—	—	—	—	2+	2+	2+[b]
	72	6+	6+	6+	6+	2+	4+	6+	6+	6+	6+
0.02 mg. NaSD	24	2+	→								2+[c]
	72	6+	→								5+
0.02 mg. NaSD 0.2 cc. NRS	24	2+	1+	—	→						1+[d]
	72	2+	1+	1+	→						4+
0.02 mg. NaSD 0.5 cc. NRS	24	—	→								1+[e]
	72	—	→								4+

[a] 10^8 *Br. abortus* added to each dilution.
[b] Control tube of medium also contains 0.5 cc. NRS.
[c] Control tube of medium also contains 0.025 mg. NaSD.
[d] Control tube of medium also contains 0.2 cc. NRS and 0.02 mg. NaSD.
NRS = Fresh, normal rabbit serum. NaSD = Sodium sulfadiazine. — = no visible growth. + = degree of growth.

mals performs in an antibody-complement system when blood serum is collected and examined shortly after infection is produced and/or thirty days or more thereafter.

The data show that the growth-inhibiting antibody for Brucella organisms can be demonstrated in low dilutions of blood serum of guinea pigs seven days after infection. A sufficient amount of fresh serum complement must always be added to each dilution to obtain this effect.

Shortly after the seventh day of infection, the Neisser-Wechsberg prozone growth phenomenon begins to manifest itself in the low dilutions of the serum. That is, no inhibition of growth of the added organisms occurs in the low twofold dilutions. Beyond the growth zone, inhibition of growth occurs in several succeeding dilutions during the first twenty-four hours of incubation. The width of the prozone and inhibition zone depends upon the concentration of specific antibody in the serum. As the period of incubation increases, the zone of growth inhibition becomes less and less and finally disappears completely. It has also been noted in the course of this study that the range of the growth zone in low dilutions of serums from infected animals usually extends beyond the agglutinin titers of the serums.

If the antibody-complement system, and the way in which it performs *in vivo*, plays an important rôle in controlling the progress of brucellosis or enters into the mechanism of recovery, it might also be a factor, as the data in table 1 suggest, in governing the therapeutic action of a sulfonamide, such as sulfadiazine. This supposition was resolved by performing the experiments which are to follow.

EXPERIMENTAL

Effect of Brucella Serum Antibody on the Activity of Sulfadiazine.—A large number of experiments have been performed which were designed to determine whether the activity and performance of the serum antibody-complement system of Brucella-infected guinea pigs, in the presence of sulfadiazine, coincided with therapeutic activity or lack of activity. Guinea pigs, which had been injected once with several times the infective doses of *Br. suis* cells, were bled aseptically by cardiac puncture on the seventh day, before and after feeding 0.25 Gm. of sulfadiazine for twenty-four hours.

Twofold dilutions of the serums were made in 5 cc. of the liquid culture medium previously described, and to these were added sufficient fresh rabbit serum as complement, sodium sulfadiazine, if it was not already present in the serum when collected, and *Br. abortus* cells. The tubes were then shaken and incubated as previously mentioned. The results, typical of several titrations of this nature that have been made, are illustrated in table 4.

The blood serum of the guinea pig which had been infected for only seven days and which showed no prozone growth phase in the dilutions used became bactericidal in test tube dilutions up to 1 : 80 in the presence of 0.02 mg. of sodium sulfadiazine (dilution 1 : 2.5 x 10^5). This concentration of the drug, when added to the medium alone, has little if any bacteriostatic effect on the growth of Brucella organisms.

The results obtained with the serum from guinea pig 2 in this experiment show that sulfadiazine increases the activity of blood

TABLE 7—Effect of Sulfadiazine on Normal or Immune Serum When Used Simultaneously on Brucella Suis and Brucella Melitensis Infection in Guinea Pigs

Group No.	Amt. of SD fed per pig[1]	Amt. of rabbit serum injected[2]	No. treated / No. controls	No. treated, infected / No. controls, infected
1[a]	0.2 Gm. daily for 7 days	0	10 / 10	10 / 10
2[a]	0.2 Gm. daily for 7 days	normal 2 cc.	20 / 20	5 / 19
3[b]	0.2 Gm. daily for 7 days	normal 2 cc.	10 / 10	0 / 9
4[a]	0.2 Gm. daily for 7 days	immune 1 cc.	10 / 10	3 / 10
5[a]	None	normal 2 cc.	10 / 0	10 / 0

[a] 10^9 *Br. suis* injected thirty days before treatment.
[b] 10^8 *Br. melitensis* injected thirty days before treatment.
[1] Drug mixed with 200 Gm. of dry feed daily.
[2] Serums injected intraperitoneally twenty-four hours after initial feeding. Serums used were collected one month prior to use and stored at 4 C.

serum to the same extent *in vivo* as it does *in vitro*. The concentration of the free drug in the serum at the time of collection was 4 mg./100 cc. A 1 : 10 dilution of this amount of the drug (the first dilution used in the titration) alone will not inhibit the growth of *Br. abortus* for twenty-four hours.

A series of experiments, similar to those just described, was conducted on a group of guinea pigs on the thirty-third day after inoculation with *Br. suis*. These experiments were designed to show: (1) the occurrence of the prozone phenomenon, which usually can be demonstrated at this time in low dilutions of serum from infected guinea pigs; (2) that prozone growth still occurs in low dilutions of the serums after the addition of both sulfadiazine and complement; and (3) that it is possible to prevent the zone growth in the low as well as high dilutions of the same or similar serums by the addition of normal rabbit serum and a small amount of sulfadiazine. The antibody titration results obtained on the serums of 3 guinea pigs, and which are representative of all the others in this series of experiments, are shown in table 5. The antibody activity of the blood serum from guinea pig 1 was determined in test tube dilutions in the medium without and with the addition of sodium sulfadiazine. Guinea pigs 2 and 3 were fed the drug daily for three days before bleeding. In addition to feeding the drug to guinea pig 3, it was injected intraperitoneally with 2 cc. of normal rabbit serum. Neither the addition of a constant amount of sodium sulfadiazine to test tube dilutions of the blood serum or the presence of the free drug in the blood at the time of collection had a marked effect on the prevention of the prozone phenomenon. There was, however, a considerable shift to the right in the bacteriostatic activity of the serum when the drug and complement were present.

No prozone growth occurred in the low dilutions of the serum in the presence of 0.5 cc. of fresh, normal rabbit serum, or after the intraperitoneal injection of normal rabbit serum (not necessarily fresh). This enhanced action does not occur in test tube without the addition of a sufficient volume of complement and a small amount of sulfadiazine. The amount of the drug required to bring about bactericidal action need not be more than 0.001 mg. per cubic centimeter of medium. The addition of 0.5 cc. of heated rabbit serum instead of fresh serum to descending dilutions of the antibody-containing serum and sulfadiazine causes no change in the prozone effect nor does it cause a shift to the right in bacteriostatic activity.

A typical example of a pronounced growth prozone effect caused by blood serum from a Brucella-infected cow, and the effect of the addition of normal rabbit serum and sodium sulfadiazine, separately or together, on the zone is illustrated in the data set forth in table 6. Neither the addition of 0.5 cc. of fresh rabbit serum nor 0.004 mg. per cubic centimeter of the drug separately reduced the growth prozone to any extent. On the other hand, the presence of the two agents together completely prevented its occurrence. All dilutions of the serum up to 1 : 4 x 10^6 to which the agents were added not only prevented the multiplication of the added bacteria but killed them as well.

Therapeutic Value of Sulfadiazine and Normal or Immune Serum on Brucella Suis Infection in Guinea Pigs.—The therapeutic significance of the *in vitro* results described in the previous experiment was determined by maintaining or prolonging the same effect *in vivo* in guinea pigs that had been infected with either *Br. suis* or *Brucella melitensis* for thirty days. The experiment was conducted on five groups of 10 pigs each, with corresponding groups of control pigs. Each of the five groups was kept in a separate cage and fed daily for seven days 2 Gm. of sulfadiazine (0.2 Gm. per pig) mixed in 200 Gm. of dry finely ground feed. At the end of the first 24-hour feeding, groups 2 and 3 were injected intraperitoneally with 2 cc. of normal rabbit serum. At the same time, group 4 was injected in the same way with 1 cc. of immune serum from a rabbit. This serum was collected from a rabbit four days after receiving one intravenous injection of 1 mg. of bacterial cells of a mucoid growth phase of *Br. suis*. Dilutions of this serum up to 1 : 160, in the presence of rabbit serum complement, inhibited the growth of *Br. abortus* for twenty-four hours. There was no prozone growth in any dilution of the serum.

The guinea pigs in each group were killed twenty-seven days after the initial feeding of sulfadiazine and examined for gross and bacteriologic evidences of infection. The results of this experiment are set forth in table 7. Those pigs in which *Br. suis* could not be detected by culture methods on autopsy showed only slight microscopic changes in their spleens. The serum agglutination titers decreased from 1 : 1,280 to 1 : 25. No histologic examinations of the tissues were made. The organs of the 5 pigs in each of the two groups that were injected with rabbit serum were not found extensively infected. Cultures of the spleens showed a few colonies of *Br. suis*.

The data furnish convincing proof that rapid and complete termination of experimentally produced brucellosis in guinea pigs can be achieved by the use of agents which prevent the occurrence of the prozone antibody phenomenon *in vivo* and permit a potentially possible bactericidal state to manifest itself. The results obtained in this experi-

ment are a practical demonstration of the thesis that the therapeutic activity of sulfadiazine in brucellosis is due, not to its direct action on the organisms, but to the formation of a sulfadiazine-antibody-complement complex. The activity of the complex is governed by the presence or absence of the prozone phenomenon.

DISCUSSION

The foregoing data represent repeated experiments that have been performed during this investigation in order to resolve the temporary bacteriostatic activity of sulfadiazine and of the antibody-complement system into one which not only possessed marked bactericidal properties for the species of Brucella in test tube, but in the infected animal as well. It has been shown that the maintenance of a bactericidal state *in vivo* for a sufficient length of time will bring about a rapid and complete termination of Brucella infection in guinea pigs.

Two hypotheses may be advanced to account for the bactericidal action that is brought about by the presence of sulfadiazine and fresh, normal rabbit serum in culture medium or in the blood of an infected animal. The first, and of course the most obvious, is that the action is an additive one. The data in table 1 show that the sodium salts of many of the sulfonamides will, in certain concentrations, inhibit the growth of Brucella organisms for a short time. It has also been demonstrated that, in experiments not included in this study, undiluted, fresh serum from supposedly normal rabbits will inhibit the growth of Brucella for an incubation period of twenty-four hours. A dilution of 1 : 10 of rabbit serum alone, such as was used in the titrations set forth in table 1, did not retard the growth of Brucella organisms in a suitable culture medium. It would then appear doubtful whether this amount of serum, in the twofold dilutions of the sulfonamides, contained sufficient antibody to bring about an additive action that was bactericidal in nature. An alternative explanation for the increased activity of the sulfonamides in the presence of fresh normal serum, or in the presence of specific antibody and complement is supported by experimental evidence; namely, that there occurs a reversible union between sulfadiazine and the antibody-complement complex and through this union, there is formed a highly bactericidal agent for Brucella organisms.

If the growth-inhibiting antibody in serum and the way in which it acts in a test tube is not involved in the action of sulfadiazine on Brucella organisms, one should always be able to demonstrate *in vitro* bacteriostatic and bactericidal action of the drug in the presence of blood serums from infected guinea pigs, regardless of the duration of infection. One should also be able to show that it has therapeutic action on *Br. suis* infection in guinea pigs thirty days after initial infection, as well as seven days after initial infection. Such action has never been demonstrated in either *in vitro* or *in vivo* experiments during this study.

The experimental data show quite conclusively that the way in which the antibody-complement system of an animal performs during the course of the disease also determines, to a great degree, the therapeutic effect of sulfadiazine on the disease. In other words, the test tube bactericidal and the *in vivo* therapeutic effects of sulfadiazine on Brucella organisms appear to be connected in some manner with the activity of the antibody-complement system. If a prozone growth effect is demonstrable in blood serum to any great degree, its occurrence can be prevented by the presence of a sufficient amount of fresh, normal rabbit serum and a small amount of sodium sulfadiazine solution. When these are added to test tube dilutions of the serum or are introduced into the blood of infected guinea pigs, a highly bactericidal action is produced. Rapid and complete termination of brucellosis in guinea pigs can be brought about when a bactericidal system is maintained in the blood of Brucella-infected guinea pigs for a sufficient length of time by the combined action of sulfadiazine, specific antibody, and complement.

The prozone growth phenomenon does not appear to be due to the blocking of complement, but to the absence of a complement-binding or reactive group in a minor

portion of the antibody protein molecules. The results of *in vitro* titrations indicate that the nonreactive protein molecules may appear in serum soon after infection occurs along with complement-binding antibodies. The molecules increase in number as the antibody increases, but not to the same extent. By making serial, twofold dilutions of a serum in which both are present, the nonreactive molecules are reduced in number to a greater degree than are the reactive ones. Apparently, the viability of the bacterial cells that combine with the incomplete antibody molecules is not affected by complement. This would account for the rapid growth of the added organisms in the low dilutions of a serum containing a large number of incomplete antibodies. The chance of incomplete antibodies combining with bacterial cells is reduced and that of complement-binding antibodies increased by serial dilution because of the ratio in which both are present in serum.

It has been demonstrated repeatedly in this study that the presence of a small amount of sodium sulfadiazine and normal serum in a serum containing nonreactive antibody molecules, converts the latter into reactive ones, *i.e.*, a bactericidal complex.

A procedure, similar to one used in the treatment of infected guinea pigs, is now being studied to determine its therapeutic value in the treatment of human and bovine brucellosis.

SUMMARY

Experimental data are presented in the foregoing experiments which show that certain of the sulfonamides form a highly active bactericidal complex with the specific antibody-complement system. This complex will prevent the growth of and kill *Brucella abortus, suis,* and *melitensis in vitro* and *in vivo.*

It has been shown that the effectiveness of this complex in killing bacteria in a test tube or in the body of an infected animal was governed by the number of inactive complement-binding antibody molecules present in blood serum.

A procedure has been developed which either counteracts the action of inactive antibody molecules or possibly converts them into active ones.

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Motility of Bovine Spermatozoa

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MOTILITY, concentration, and morphology are the three factors of fundamental importance in evaluation of semen quality. This study covers the initiation of motility, the various types exhibited, abnormal morphology, and the grading of semen samples on the basis of motility.

INITIATION OF MOTILITY

During their long passage from the seminiferous tubules to the ampullae, sperm show little or no motility, while the ejaculated sperm are in a state of violent activity. The development of this function occurs quite early, and even in the seminiferous tubules these developing cells are potentially motile. The quiescent, ripening sperm at any part of their passage from the testes to the ampullae can be readily made to demonstrate motility by using dilution techniques. The diluted testicular sperm exhibit a convulsive type of motility with very little progression. A more active type of motility is manifested in diluted samples from different parts of the epididymis.

In the ampullae, motility is first observed undiluted, but this is greatly improved by diluting with warm physiologic saline. Probable causes of the suppression of motility are crowding of a large number of sperm per unit area and the viscosity of the medium. This is supported by the markedly reduced sperm concentration in the ampulla compared to the epididymis. (*See* data on 5 bulls in table 1.)

The low content of the epididymal fluid in Cl-ions and electrolytes generally, its acid reaction, and the lower temperature of the scrotum also retard activity.[1]

In ejaculated sperm, Milovanov[1] attributed the sudden development of activity to the high content of electrolytes, particularly sodium chloride, and the alkaline reaction of accessory secretions. Our observations show that normal saline has a distinct stimulating effect on epididymal and on ejaculated sperm, being superior in this respect to 5 per cent glucose solution. Addition of the secretions of the seminal vesicles to the epididymal sperm *in vitro* elicited very little activity response. Walton[3] thought the function of the accessory secretions was mainly mechanical, giving bulk to the ejaculate and rendering the peristaltic contractions of the urethra more effective in forcing the sperm to the exterior. The accessory secretions serve to disperse the sperm and this alone stimulates activity, but other factors should not be overlooked. Ejaculation is accompanied by muscular contractions of the epididymides, vasa deferentia, and ampullae, the admixture to sperm at the neck of the bladder of prostatic and seminal secretions, the whole being rapidly driven to the exterior by the powerful peristaltic contractions of the urethra. The orgasm waves of contraction that start in the epididymides are multiple and rhythmic, following one another in quick succession, which adds to the stimulus. The urethra has a higher temperature, and its lumen is reduced both by pressure of the engorged cavernous tissue and contraction of the urethral wall. These multiple processes are well timed and synchronize harmoniously, producing a further stimulatory effect on sperm motility.

The structure and motility of mammalian sperm are associated with the capacity for rheotaxis, *i.e.*, orientation and movement against a flow of liquid. Their movement is activated by a contrary flow and, according to Yamane and Ito,[4] the stallion sperm are able to increase their rate of movement from 87 to 200 μ per second against a current. This has frequently been noticed in our work. When, after storage, motility in a sample of semen is low, applying a little pressure on the coverslip and releasing it greatly stimulates motility due to the current set up.

Zeller[5] suggested that the motility of the human sperm is dependent upon two enzyme systems, a diamine oxidase and a monamine oxidase, found mainly in the seminal fluid. Weber[6] noted that when the motility of bull sperm had ceased in storage it could be restored with isotonic salt or glucose solution. We have been able to confirm this both with straight semen and that diluted with egg yolk buffer.

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MECHANISM OF MOTILITY

Milovanov[1, 2] describes sperm movements in farm livestock as resulting from unilateral strokes of the tail in one plane, with rotation round their long axis owing to the asymmetrical structure of the head. Throughout development, the head of the bull sperm is an elongated oval when viewed on flat. It is slightly biconcave when viewed on edge and, in this position, is of about the same thickness as the connecting piece of the tail and does not appear to be asymmetrical. Very high dilutions of semen were used in our study of sperm motility, so that the movement of individual sperm could be carefully observed and their path followed. Our observations lead us to conclude that sperm movement is effected by bilateral lashings of the tail in a horizontal plane, the amplitude being inversely proportional to the vigor of motility. In other words, the lashings of the tail are frequent and powerful, but brief (low amplitude) when motility is maximal whereas, with decline in vigor, the amplitude of lashing increases and the force and frequency decrease, indicating a greater effort on the part of the sperm to effect locomotion. It was also noticed that in maximal motility the sperm always travelled on edge, since this is the position offering minimum resistance, but no rotation on the longitudinal axis was observed. The head of the sperm has considerable freedom of movement about the neck, which is rather flexible, and while the tail is the propelling mechanism, the head acts as the rudder and determines the direction in which the sperm move. In maximal motility sperm move in a linear fashion, but frequently change direction. With declining vigor, the direction of movement becomes erratic. The connecting piece does not seem to take any part in the lashing movements. Cody[7] believes that the connecting piece acts as the motor of the sperm and, if the head is severed, the tail continues to swim in a straight line. We have occasionally observed headless sperm exhibiting movement which was rather aimless and weak. Cody[7] also states that injury to the tail piece caused loss of motility, but the activity impulse is strong and not ordinarily destroyed even by serious abnormalities of the tail. Thus, sperm with a complete bending of the tail at the posterior end of the connecting piece maintain motility for a great length of time. Popa[8] suggests that the posterior centriole may be the source of the power of motility. Herman and Swanson[9] investigated the interchange of sperm and seminal fluid of different bulls and found that the motility after ejaculation was more dependent on a property of the sperm themselves than upon the type of seminal fluid in which they were suspended.

It is commonly believed that cessation of motility under normal conditions is a gradual process, and once movement stops it is never regained.[7] Our observations point to the contrary. If the path of a sperm exhibiting maximal motility is carefully followed, it will be seen to proceed, for the most part, in a linear fashion and finally slow down or stop rather abruptly. It then shows convulsive movements and comes to rest, occasionally exhibiting twitching movements. When such resting sperm are pushed aside by some actively motile sperm, they are stimulated to motility for a brief period. Still another evidence that quiescent sperm can regain motility is that small amounts of a 0.1 per cent solution of strychnine sulfate will cause resumption of motility.[10] This can also be done with other techniques previously described and demonstrates that all nonmotile sperm are not dead.

The rate of sperm movement greatly varies with species, vigor, and the medium. The approximate rate of movement in millimeters per minute has been reported as follows: stallion 5.2,[4] bull 4.02, ram 3.00, and dog 2.58.[11] Phillips and Andrews[12] found the average speed of ram sperm in Ringer's and normal salt solutions was 4.6 mm./minute. Bretschneider[13] reported that vigorous shaking of bull semen for three minutes destroyed motility.

TYPES OF MOTILITY

Several workers have tried to classify the various types of sperm motility. Reynolds[14] described five types for human sperm: rapid vibratile, slower vibratile, bunting, rotary, and pendulum motion. Walton[3] recognized three types: progressive, rotary, and oscillatory. In addition to these classifications, other workers have used different terms to describe various other types of movements.

We have classified motility of sperm into three main types, depending on the vigor and direction of movement.

1) *Maximal Motility.*—This is the most active type of motility in which the progression is linear. The head and connecting piece are in a straight line, except for the occasional postural changes in the head. The lashings of the chief piece are quick, brief, and powerful, and the sperm head assumes the on-edge position in first grade maximal motility. As the sperm become weaker, it is more difficult to maintain the on-edge position and hence, they topple over to the on-flat position but try to return to the on-edge position. This alternating position is the characteristic of a second grade maximal motility. As the sperm get still weaker, they are no long-

er able to maintain the on-edge position and remain in the on-flat position throughout their progression, and this constitutes the third grade maximal motility. Although these are minor differences in the type of motility exhibited, they are easily recognizable and have a bearing on the vigor of sperm and the speed of their movement.

2) *Circular Motility.*—This is an abnormal type, evidencing considerable vigor and endurance, exhibited by sperm with abnormalities of the tail. Those sperm which show a partial bending of the tail at the posterior end of the connecting piece have their whole length bent in a somewhat semicircular fashion. In such cases, the body of the sperm cell itself forms part of the circumference of the circle along which it moves.

TABLE I—Relative Concentration of Sperm in the Tail of Epididymis and Ampulla

	Ampulla No./mm.^{3}	Tail of epididymis No./mm.^{3}
Guernsey I	1,280,000	2,020,000
Guernsey II	2,370,000	5,180,000
Hereford I	1,240,000	2,290,000
Hereford II	2,160,000	5,360,000
Hereford III	4,510,000	9,310,000

In other cases, the bend of the tail at the end of the connecting piece is complete, forming a loop. These sperm exhibit slight vibrations as they move around the circumference of a circle, due to the interference with the lashing of the tail, but do not necessarily exhibit circular motion.

3) *Convulsive Motility.*—This class includes all other types of motility and is indicative of decline in vigor. It is characterized by the erratic course taken by the sperm, the great difficulty in effecting locomotion, and the poor progression made in spite of the violent efforts. The range of variation is great, including sperm which are practically stationary but continue to lash their tails, as well as sperm which show a fairly active, serpentine movement.

These various types of movements are an index of vigor. Under routine artificial insemination, maximal motility is desired.

Motility and Fertilizing Capacity

Motility has been extensively used in judging semen, and many workers believe it to be the best index in the evaluation of semen quality. The view that sperm lose their fertilizing ability long before the loss of motility has been held for a considerable time. It is reasonable to expect sperm with poor motility to be incapable of fertilization because they fail to reach the ovum, rather than due to any loss of fertilizing capacity. Chabibullin[15] found in ram semen marked variations between the percentage of sperm motility and the conception rate in stored semen. Underbjerg and Davis[16, 17] presented evidence that in stored semen good motility is not identical with fertility. The higher conception rates obtained with semen samples of better motility is indicative of its importance in semen evaluation.

Dilution Rate.—Chang,[18] working with rabbits, found that when the same number of spermatozoa (30,000 to 44,000) were suspended in 0.1, 0.4, and 1 cc. of saline, respectively, and cervically inseminated into superovulated does, the mean percentage of cleavage of ova was 2.75 with 1 cc., while with 0.1 cc. it was 27.0. He suggests that these results might be due to the detrimental effects of chemical ions, oxygen tension, etc., at the higher dilutions, or the undue dilution of certain beneficial substances resulting in the loss of fertilizing capacity, or the ability of the rabbit cervix to take up only a very small amount of fluid for inseminations.

We have investigated the effect of various dilutions of semen with saline and egg yolk buffer on sperm motility. At 1:400 dilution, most of the cells were immotile in about two hours when saline was used as diluent, while with egg yolk buffer, motility was retained as long as in control samples diluted 1:10 or 1:15 with egg yolk buffer. When the same samples of semen were diluted serially from 1:10 to 1:400 with saline, the longevity of the sperm was greatly reduced at the higher dilutions. At the higher dilutions, the sperm are so greatly stimulated that they rapidly exhaust themselves. It is thus possible that in Chang's experiments the low percentage of cleaved ova at the higher dilutions was due to an actual reduction in the percentage of live and motile sperm. The probability of similar dilutions of semen being used in the artificial insemination of livestock is rather remote. Salisbury[19] used semen diluted 1:40, 1:60, 1:80, and 1:100 with egg yolk buffer for inseminations and found that the highest average efficiency of conception was obtained with the highest dilution rate of 1:100 (79.4%), the average

number of sperm being about 12,861,000/cc. of diluted semen.

EXAMINATION AND EVALUATION OF SEMEN

A simple and very practical method of preparing a sample is to place a small drop of undiluted semen on a clean glass slide and examine it; four to five samples may be examined on one slide. There is no special advantage in using a coverslip. When coverslips are used, several precautions should be taken. The coverslip should be absolutely clean to avoid introduction of any chemical agents which might inhibit sperm motility. A fair sized drop of semen should be taken so that the coverslip is not pulled too close to the slide and thereby interfere with sperm movement.

It is advantageous for all semen examinations to be carried out at a constant temperature, both in the laboratory and in the field, and 100 F. is the most suitable for this purpose. With the use of a warm stage, sufficient time should be allowed for the drop of semen to reach the desired temperature, care being taken not to overheat. The Russians devised a numerical system of grading, reported by Walton,[3] based upon the proportion exhibiting the various types of motility. This method was followed by the Missouri workers (Herman and Swanson[9]) with slight modification. Gunn[20] has proposed a more complicated means of classification. Various other workers have expressed grade in terms of percentage of motile sperm.

The grade of motility is dependent not on the total number of motile sperm, but on the total energy output of the whole, causing a certain visual picture under the microscope. In our laboratory we have used the following grades to express the intensity of motility presented by a sample of semen when examined under a magnification of 250, using a warm stage set at 100 F.

Good.—About 80 per cent of sperm are alive and most of them in maximal motility; there is profound disturbance in the microscopic field, presenting the appearance of a whirlpool with the rapid formation and dispersal of waves and eddies.

Medium.—About 65 per cent of sperm are alive and a large proportion of them in maximal motility; the formation of waves and eddies is not pronounced. Disturbance in the microscopic field is very marked.

Fair.—About 50 per cent of sperm are alive and only a small proportion of them exhibiting maximal motility. Disturbance in the microscopic field is not pronounced.

Poor.—About 35 per cent of sperm are alive and very few or none in maximal motility. Disturbance in the microscopic field is poor.

Dead.—There is absolutely no movement in the microscopic field.

This grading system can be used after dilution with egg yolk citrate buffer, provided dilutions are not too high.

The percentage of live sperm is ascertained with opal blue eosin staining mixture.[21] This can be checked with actual counts in undiluted semen by using a warm stage, the hemocytometer slide, the red blood cell pipette, and physiologic saline at about 105 F. The hemocytometer slide is kept on the warm stage. The semen is diluted as usual with the warm physiologic saline and, after discarding the first two or three drops from the pipette, a drop is run under the coverslip. Allowing sufficient time for the nonmotile sperm to settle down, the total number of nonmotile sperm in five large squares is rapidly counted. This number multiplied by 10,000 gives the total number of nonmotile sperm in 1 mm.^{3} Then the percentage of nonmotile sperm can easily be calculated from the total sperm count previously obtained with the use of 3 per cent saline as a killing agent. Variations in these two methods occur but are consistent in that the opal blue eosin method gives a higher percentage of live sperm, because nonmotile sperm still living do not take the stain.

Our observations reveal that samples of good, initial motility retain their motility for a longer period than samples of poor, initial motility.

SUMMARY

Motility is an early characteristic of the developing sperm, which are kept in a state of anabiosis by environmental factors, mainly the low electrolyte content of the epididymides, viscosity of the medium, low temperature in the scrotum, and space limitations. Though motility can be induced in sperm from the deeper regions of the reproductive tract, it is not maximal. Sperm motility is normally initiated in the ampullae. The violent activity of the ejaculated sperm, is not merely the outcome of admixture with the accessory secretions rich in electrolytes, but is actually the end result of a series of functions which harmoniously synchronize at the time of ejaculation.

The actual propulsion of the sperm is brought about by bilateral lashings of the chief piece in one plane, while the head directs the course to be taken. The connecting piece does not appear to take any active part in locomotion, and the posterior centriole probably acts as the locomotor center.

In maximal motility, sperm move with ease and little effort, while convulsive motility in weaker sperm is characterized by violent efforts to effect locomotion. Motility may be regained after it stops, but such motility is not prolonged.

The dilution effect which causes resumption of motility is passive and distinct from the stimulatory effect of dilute solution of strychnine sulfate.

The types of motility exhibited by the sperm vary widely, and a method of classifying them has been suggested which is based on the vigor and direction of movement. Though motility has been criticized as an inadequate index of the fertilizing capacity of the sperm, it is still the best criterion.

The examination of semen samples for motility cannot be regarded as an unskilled job and, unless the proper techniques are used, paying due attention to all details, it might lead to misunderstanding between the field inseminator and the laboratory worker. A simple system of grading semen samples based on the amount of disturbance in the microscopic field has been discussed.

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Hemolymph Nodes in Small Ruminants

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AS IS GENERALLY known, ruminants possess ordinary lymph nodes and also another kind of similar bodies, round or oval, and dark or light red. These latter represent a special group of lymph nodes and may be designated blood-lymph or hemolymph nodes.

ANATOMIC ASPECTS

Like the spleen, these nodes are directly connected with the blood circulatory system. Externally, they appear like supplementary lymph nodes stained red (of round or oval shape and apparently rather tense or distended). They do not possess the characteristic afferent and efferent vessels of ordinary lymph nodes. Their distribution (number and location) is variable. Generally, they can be found at any place in the organism. However, they are frequently associated with ordinary lymph nodes in the large cavities (thoracic, abdominal, pelvic). Sometimes they are found in localized groups without association with ordinary lymph glands.

Their specific location in the splanchnic cavities are as follows: in the thoracic cavity, along the vertebral column near the aorta; the abdominal cavity, in the adipose tissue surrounding the kidneys, around the large blood vessels (aorta, vena cava, portal vessels) at the hilus of the liver, at the rumen, and occasionally between the loops of the intestine; the pelvic cavity, especially at the entrance (retroperitoneal area).

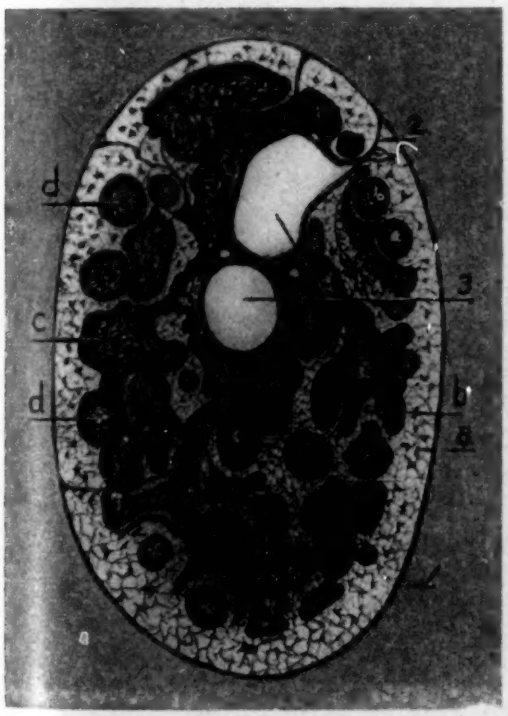

Fig. 1—Cross section of a hemolymph node of the goat. The entire sinus system has been emptied by washing. 1 = capsule; 2 = a trabecula originating from the capsule and extending into the gland; 3 = cross section of a blood vessel; a = marginal sinus; b = intermediate sinus; c = red pulp; d = white pulp forming secondary nodules showing their relationship to the red pulp.

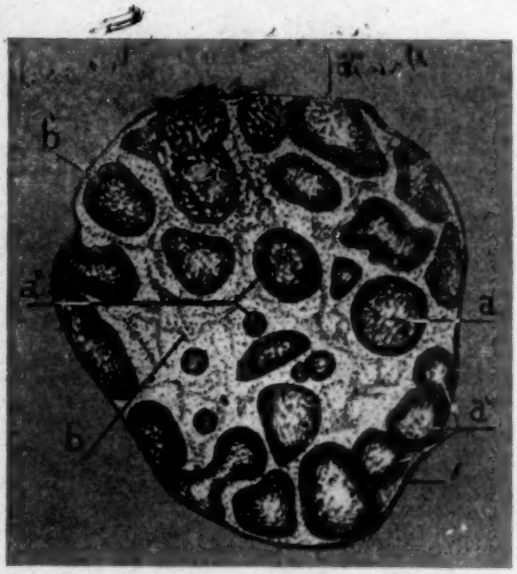

Fig. 2—Cross section of a hemolymph node of the sheep. 1 = capsule; a = white pulp forming secondary nodules; a ''' = secondary nodules in direct contact with the capsule; a ' = centrally located nodules; b = the red pulp; b ' = a thin mass of red pulp which lies between the capsule and the secondary nodule.

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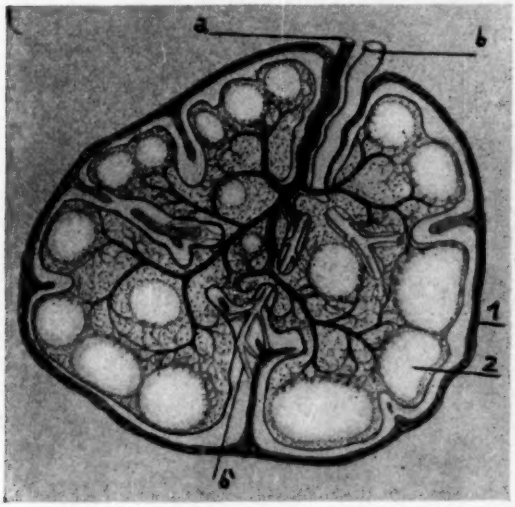

Fig. 3—Vascularization of a hemolymph node. a = artery; b = vein; b' = vein traversing the intermediate sinus; 1 = capsule; 2 = secondary nodule.

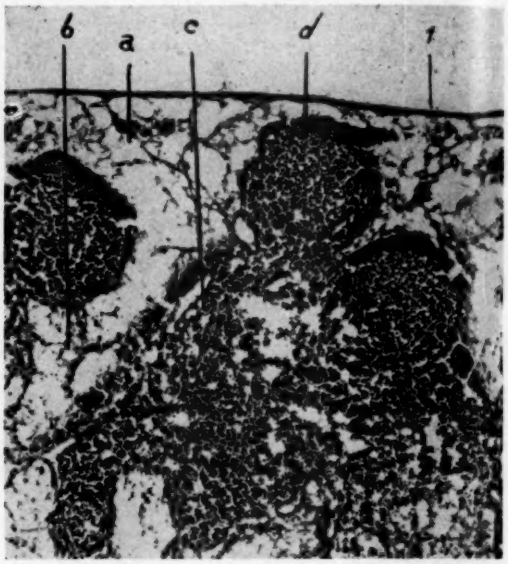

Fig. 5—Section of hemolymph node of the goat. 1 = capsule; a = marginal sinus; b = intermediate sinus; c = red pulp; d = a secondary nodule.

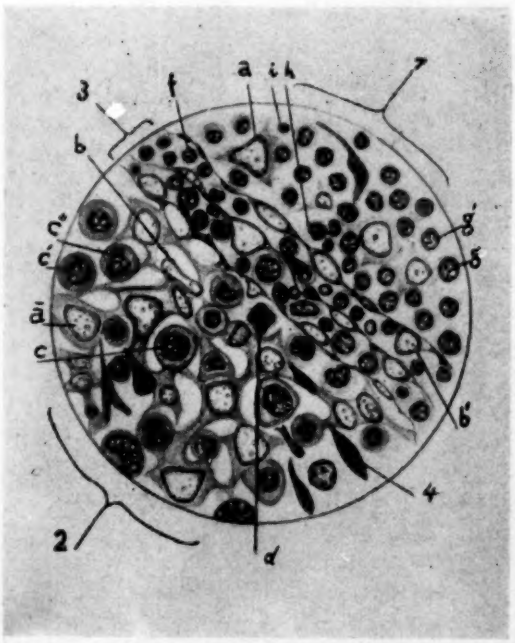

Fig. 4—Details of structure of red and white pulp. 1 = red pulp; 2 = white pulp (secondary nodule); 3 = intermediate zone between red and white pulp in which the reticular tissue forms a small meshed, dense network; 4 = trabecula; a = reticulocyte in the red pulp; a' = reticulocyte in syncytium; b, b' = reticulocytes of endothelial character; c, c' = lymphoblast; c'' = lymphoblast with a daughter nucleus; d = monocyte; f = lymphocyte; g = lymphoblast; g' = lymphocyte; h = myelocyte; i = erythroblast.

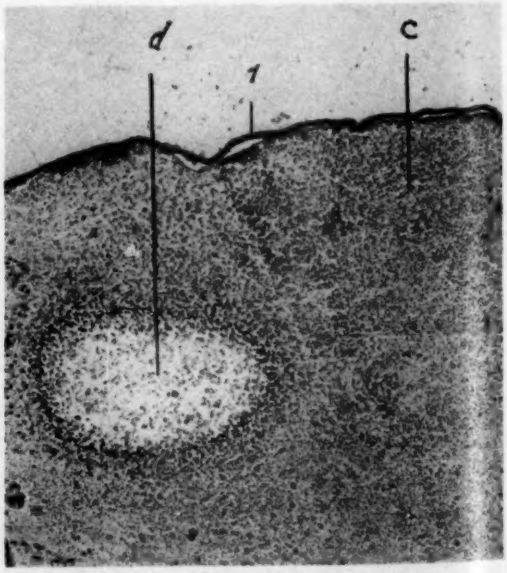

Fig. 6—Section of hemolymph note of the sheep. 1 = capsule (it is evident that the parenchyma touches the capsule and that the marginal sinus is but a slit); c = red pulp; d = secondary nodule.

Their size varies from that of a pinhead to that of a hazel nut.

General Structure of the Hemolymph Nodes.—The organ is almost completely surrounded by a capsule consisting of fibrous connective tissue and smooth muscle fibres. The capsule sends strong trabeculae into the glandular tissue, especially at the hilus where the blood vessels enter and emerge. These trabeculae are supposed to form the framework of the blood-lymph nodes. The parenchyma is found in the meshes of this framework as well as between the trabeculae and the capsule. Primary, secondary, and tertiary trabeculae can be distinguished. The parenchyma is separated from the trabeculae and the capsule by a system of marginal and intermediate sinuses. These sinuses are more distinct in the goat than in the sheep. Reticular connective tissue originating from the capsule and from the trabeculae pervades the entire system of sinuses and crosses into the reticulo-endothelial network of the parenchyma. Thus, the parenchyma is attached to the capsular framework of the entire gland (fig. 1a).

The parenchyma represents a lymphoreticular tissue. It is richly seeded with secondary nodules, located toward the periphery of the node in sheep and goats so that it is proper to refer to them as the cortical part of the parenchyma. This condition is especially noticeable in the sheep. It is not unusual for these nodules to be found in the center of the node (fig. 2a). The rest of the parenchyma can be regarded as medullary substance (fig. 2b). In contrast to the cortical substance, which consists of lymph nodules, the medullary substance is rich in such myeloid cells as erythrocytes, erythroblasts, and myelocytes.

Vascularization of the Hemolymph Nodes.—An artery enters the organ at the hilus and divides into a capillary system in the cortical substance. The arterial capillaries of the parenchyma open into the delicate sinus system of the medullary substance. The venous capillaries are distal to these sinuses, and their function is to return the blood. The secondary nodules of the medullary substance represent islands into which no capillaries open, the erythrocytes occasionally found there being derived from the medullary substance itself.

The extensive sinus system (marginal and intermediate) which is present between the capsule and the reticular framework is filled with venous blood.

The stroma of the medullary and cortical substances is typically reticulo-endothelial in character. Numerous lymphoblasts were found in various stages of development in the secondary nodules, (fig. 4c, c′, and 4″). The peripheral cells of the nodules are mostly mature lymphocytes which may enter the blood stream immediately or may need to undergo further maturation within the medullary sinuses.

Various representative forms of the myeloid family were found regularly in the medullary substance—erythroblasts, myeloblasts, and myelocytes (fig. 4h and i). The remaining components of the medullary substance are numerous mature and juvenile lymphocytes and erythrocytes.

The stroma of the medullary substance resembles the cortical substance, it being also reticulo-endothelial in character.

It may be concluded, then, that from a structural and cytologic point of view and considering the kinds of cells they contain and produce, the hemolymph nodes are splenoid in character.

We shall soon emphasize that, contrary to the spleen, these organs also contain myeloid blood cells. Therefore, they could be considered either as embryonic spleen or as representing an additional myeloid system similar to red bone marrow.

Structural Differences of the Hemolymph Nodes of the Sheep and the Goat.—The hemolymph nodes of the sheep possess an essentially thicker capsule which encompasses the parenchyma so closely that it is often difficult to distingush the marginal sinus, which is present even when almost unidentifiable. In contrast to this, the marginal and intermediate sinuses of the goat are so distinct that they can be plainly seen in stained and unstained section (fig. 1). Because of these wide and well developed sinuses, the parenchyma appears

to be divided into fields—it looks like an island with many fjords.

The grouping of the nodules in the pulp also differs in sheep and goats. In the sheep, the nodules are located peripherally or marginally, so that they form a true cortex beneath the capsule. In the goat, however, these nodules are found irregularly

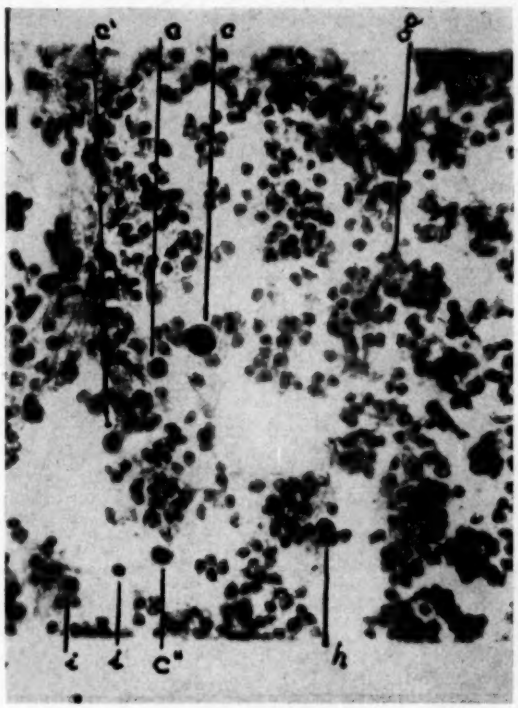

Fig. 7—The blood cells of the red pulp. c, c' = lymphocytes and lymphoblast; c" = lymphoblast with daughter nucleus; h = monocyte; i = erythroblasts.

distributed throughout the parenchyma, so that we are not justified in speaking of a cortical substance (fig. 1).

On the basis of these properties, it is immediately possible to distinguish between sections of the hemolymph nodes of sheep and those of goats (fig. 1 and 2). The differences are also reflected in the macroscopic appearance of these organs. When glands from both species are fixed in formalin, they are easily distinguishable, for the sheep glands have whitish spots and a glistening surface while those of the goat are uniformly dirty red (fig. 8b and 8a).

The difference in external appearance of the formalin-fixed glands is easily explained, the nodules of the sheep lie just under the capsule to which they are attached by fixation and shimmer through, while the capsule itself is becaming semitransparent.

The reaction can be produced in a few minutes in Carnoy's fixing fluid. (The hemolymph nodes of the sheep and the goat destined for this reaction must be dark red; the pale red or white nodes of the goat are easily confused with those of the sheep.) It is immediately evident why this glistening appearance can not be produced in the goat glands, the reason being that the wide, blood-filled subcapsular sinus system separates the parenchyma from the capsule and prevents direct action in the parenchyma through the capsule.

DISCUSSION

Because both sheep and goats are slaughtered for human food in Turkey, and be-

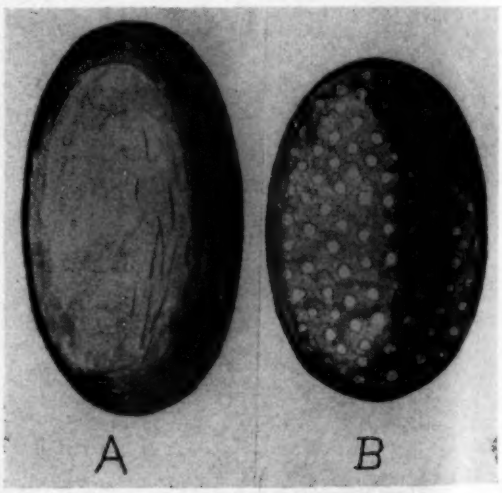

Fig. 8—The fixed hemolymph nodes of the goat (A) and the sheep (B).

cause no easy method of differentiating between the two types of meat has been known previously, the macroscopic and microscopic differences which make such a distinction definitely possible are of considerable importance to us—especially so, because these nodes are found in all parts of the body and are likely to be present in every piece of meat sold.

These organs, especially those of the goat,

offer excellent material for demonstrating the reticulo-endothelial system and have marked didactic value because they too have a sharp and glistening appearance. Such demonstrations may be prepared in the following manner: The hemolymph nodes should be fixed in 33⅓ per cent alcohol for at least one week, sectioned with a freezing microtome set at 5 to 10μ, rinsed as much as possible in distilled water, stained with hemalum or with Delafield's hematoxylin, carefully decolorized, and finally counterstained with eosin.

Another method which has produced beautifully stained sections is that which uses Mare's methyl green solution, but it is important that such sections be prestained with hemalum and then meticulously decolorized with alcohol following the methyl green staining if clear sections of value are to be obtained.

The hemolymph nodes are typically lympho-reticular in character, as we have learned. Possessing myeloid cells and erythroblasts, they must be classed with the hemopoietic organs. From this information, I have concluded that these glands are highly important in all blood diseases and all pathologic conditions of the blood-forming organs and should be examined along with the spleen whenever these conditions are being studied.

The Control of Ovine Enterotoxemia by the Use of Clostridium Perfringens Type D Bacterin

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THE IMPORTANCE of ovine enterotoxemia in the United States is evidenced by the increasing number of reports in the literature upon this subject. As pointed out by Muth[1] and Whitlock and Fabricant,[2] numerous terms have been used to describe this condition, the only difference being the age at which the disease occurs and the conditions under which it makes its appearance; *i.e.*, pulpy kidney disease, braxy-like disease, overeating disease, apoplexy-like disease.

The majority of investigators concede that the disease is due to a toxemia resulting from the production of toxin by *Clostridium perfringens* (*Clostridium welchii*) Type D in the small intestine. This organism may be found as a saprophyte in the lower bowel of sheep, particularly those which have been exposed to soil on which sheep have been kept continually. The mechanism in which the disease operates is not thoroughly understood but the following hypothesis seems logical: The ingestion of large quantities of highly nutritive feedstuffs, such as milk in the case of young, single lambs, succulent grass in the case of older lambs, and grains in the case of feeder lambs, produces an environment in which *Cl. perfringens* Type D proliferates at a rapid rate. The organism under the afore-mentioned conditions invades the small intestine and produces a small quantity of toxin and large quantities of nontoxic protoxin[3, 4] which has the unusual characteristic of being activated to potent toxin when mixed with the digestive enzyme, trypsin. The toxin thus formed is absorbed through the small intestine and produces death in the majority of instances. Generally speaking, the higher the intake of concentrated feedstuffs the greater is the incidence of enterotoxemia.

Under commercial feedlot conditions, ovine enterotoxemia is an ever present problem. Even when well controlled rations are fed, the average commercial feeder has learned to expect losses from enterotoxemia, and many such feeders are willing to sacrifice a 1 per cent overall death loss per month before becoming alarmed.

The control of enterotoxemia in foreign countries[5, 6, 7, 8, 9, 10, 11] under range and feedlot conditions has been at least partially accomplished by means of biologic agents. In this country, Muth,[1,12] using both antitoxin and toxoid prepared by his own techniques, was successful in controlling enterotoxemia under range conditions. The report of Whitlock and Fabricant[2] of Cornell University indicated encouraging, but far from successful, results in controlling enterotoxemia in feedlot lambs immunized

TABLE I—Comparative Combining Power of Clostridium Perfringens Type D Bacterin

	Bacterin (amt.)	Antitoxin (units)	Toxin, test dose	Results mice*	C.P.
1946 product	0.05 cc.	0.5	0.14	5/5	
	0.05	1.0	0.14	0/5	10
Bacterin control	0.05	...	...	0/5	
1947 product	0.05	2.5	0.14	5/5	
	0.05	3.0	0.14	0/5	50
Bacterin control	0.05	...	...	0/5	
Toxin control	...	...	0.07	5/5	

*The denominator represents the number of mice injected with each mixture and the numerator the number of mice that died.

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with imported anaculture (whole culture toxoid).

For a complete review of the literature, the reader is referred to the report of Whitlock and Fabricant[2] in which 89 references are cited.

Since the most logical approach to the control of ovine enterotoxemia would appear to be through the establishment of active immunity to the Type D toxin of *Cl. perfringens*, a preliminary investigation was conducted during the 1946 winter feeding season through the coöperative efforts of a veterinary biologic house and a commercial feeding concern. In light of the encouraging results obtained, a second rigidly controlled experiment was conducted during the winter feeding season of 1947.

MATERIALS AND METHODS

The 1946 experiment commenced Dec. 13, 1946, and had as its objective the determination of the effectiveness of alum-precipitated *Cl. perfringens* Type D bacterin (Corn States*) in reducing the overall death loss. It was conducted without altering the routine feeding operations and with a minimum of technical work.

The 1947 experiment commenced Oct. 10, 1947, and had a twofold objective: (1) to determine the actual reduction of enterotoxemia losses when judged under rigidly controlled conditions and critical laboratory diagnostic methods; (2) to force, within practical limits, the consumption of fattening elements (corn and molasses) to the immunized lambs, providing that they showed resistance to enterotoxemia. The control animals were similarly force-fed until enterotoxemia deaths became too numerous, and it was obvious that a continuation would result in undue loss. Thereafter, the ration and management of the controls was maintained in the customary manner.

The ration was hand-fed during the 1946 experiment and consisted of 30 to 45 per cent corn, 10 per cent molasses, 5 per cent soy bean oil meal, 1 per cent mineral, and the balance chopped alfalfa hay. The concentration of corn was initially 30 per cent and was gradually increased to 45 per cent by the fourth to fifth week. The quantity of feed and concentration of corn was regulated throughout the experiment by the number of deaths and the evidence of diarrhea in the lots.

*For the sake of simplicity and space conservation, the terms "bacterin" or "product" are used to refer to *Cl. perfringens* Type D bacterin (Corn States) throughout the balance of this paper.

The ration used in the 1947 experiment was the same as that in the 1946 experiment with two exceptions; the immunized sheep were fed up to 60 per cent corn, and midway in the experiment the molasses concentration for both lots was increased to 15 per cent and the corn concentration correspondingly reduced 5 per cent.

It is the practice of commercial feeders to cull all lots of lambs on feed daily and salvage the culls by slaughter, if possible. Arrangements were made in this study whereby such culled animals could be inspected and specimens obtained from them for laboratory examination.

Sheep were treated upon arrival at the feedlot by injecting 5 cc. of the bacterin. Although the term bacterin has been applied to this product, it is a whole culture toxoid, and the potency is dependent upon its toxoid content.

Injections of the bacterin were made into the tissue slightly posterior to the axillary space, and Shikles equipment was used to expedite the operation. Care was taken not to inject the product directly into the axillary space, since negative pressure is produced in this area when the forelimb is extended laterally, and air can be sucked into the subcutaneous tissues through a needle hole. Even with inexperienced handlers and no special equipment, it was possible to inoculate approximately 2,000 animals in five to six hours at a handling cost of 1½ cents per head.

Control animals in both experiments were selected indiscriminately. Adjoining lots having the same feeding space and conditions were used. Feeding operations had been carried on for many years in these lots, thus insuring natural exposure to *Cl. perfringens* Type D.

In the 1946 experiment, 1,880 lambs averaging 78.3 lb. each were injected with the bacterin, and 1,903 lambs of the same origin averaging 74.8 lb. each were left as controls. These animals were shipped from Montana where they had been semifattened on sugar beet tops and pulp.

Postmortem and laboratory examinations were not performed on the lambs removed from the experiment. However, records were kept of the comparative feed consumption and the overall loss of dead and culled animals.

In the 1947 experiment, 1,967 lambs averaging 65.7 lb. each were injected with the bacterin, and 960 of these were given a second 5-cc. dose two weeks following the administration of the first. 1,970 lambs averaging 64.6 pounds each served as controls. The animals came from the ranges of western Wyoming and could be classed as typical feeder lambs.

A total of 116 dead and culled lambs were removed during the course of the 1947 experi-

ment. Of these, 4 dead sheep were not observed or autopsied, and the tissues of 6 sheep, 4 of which were asphyxiated due to overcrowding and 2 culls removed late in the experiment because of lameness, were not tralized. Intestinal filtrate samples were frequently stored at a subfreezing temperature for two weeks or more and apparently withstood the storage with little deterioration. Only those cases in which the Type D toxin

TABLE 2—Individual Antitoxin Level of Lambs Thirty-two Days after Immunization, 1947 Experiment

Group	Total No. lambs	No. lambs showing antitoxin (units/cc. serum)											
		0	1/5	1/3	1/2	1	2	4	8	16	32	64	128
Control	20	15	1		2	1			1				
Immunized one dose	20						1	9	2	2	3	1	2
Immunized two doses	20								1	3	3	6	7

subjected to a laboratory examination. Tissues including the spleen, kidney, liver, and lung were taken from each of the other 106 animals and subjected to a bacteriologic examination for both aërobes and anaërobes. Mediums used for these purposes consisted of dextrose starch agar for aërobes and Brewer medium as modified by Vawter and Records[12] for anaërobes. The intestine was routinely cultured upon SS agar (Difco) for the first 35 cases but, in light of the absence of Salmonella carriers, this procedure was used only in suspected cases of salmonellosis.

The contents of the small intestine were collected from all of the 74 sheep showing lesions of enterotoxemia and from 29 of the sheep showing lesions other than enterotoxemia. These specimens were examined for the Type D toxin of *Cl. perfringens* in the following manner: Equal volumes of saline and intestinal contents were mixed and centrifuged at 2,500 r.p.m. for one half-hour. The supernatant fluid was filtered through a Seitz E. K. disk and the filtrate tested for sterility. Injections of 0.2 cc. of the filtrate were made intravenously into Swiss mice which were observed for eighteen hours before concluding the test. If the filtrate proved to be toxic, it was then mixed with an equal volume of standard Type D antitoxin and allowed to stand for one hour at room temperature. Injections of the mixture were then made intravenously into mice to determine if the toxin had been neutralized. Intestinal filtrate samples were frequently stored at a subfreezing temperature for two weeks or more and apparently withstood the storage with little deterioration. Only those cases in which the Type D toxin could be demonstrated by the foregoing technique were positively diagnosed as enterotoxemia. Specimens were obtained fresh whenever possible, but not infrequently animals were dead an estimated twelve to eighteen hours before an autopsy was performed.

SEROLOGIC TECHNIQUES

The specific standards of measure that follow were arbitrarily set to facilitate antitoxin determinations of sheep serums and combining power values of the bacterin.

Standard Toxin. — A dry, cream-colored powder was obtained by extracting cell-free filtrates containing toxin and protoxin[3, 4] by the simple ammonium sulfate method used by MacFarlane and Knight.[4,7] One m.l.d. of standard toxin was 0.002 mg.

Minimal Lethal Dose (m.l.d.)—This was the smallest quantity of activated toxin necessary to kill, when injected intravenously, 4 of 5 Swiss mice weighing 17 to 19 Gm. each. Activation was accomplished by dissolving the toxin in saline, using 2.5 mg. of trypsin per milligram of toxin and incubating the mixture for forty-five minutes at 37 C.

Test Dose (*T.D.*).—That quantity of activated standard toxin (0.03 mg.) representing at least 15 m.l.d.'s completely neutralized by 1 unit of antitoxin was the test dose.

Standard Antitoxin.—Horse serum containing 500 units of antitoxin per cubic centimeter was used.

TABLE 3—Antitoxin Titer and Resistance to Toxin of Lambs Sixty Days after Immunization, 1947 Experiment

Group	No. lambs injected	Amt. of toxin mouse m.l.d.	Results*	Individual antitoxin levels** units/cc. serum
Control	2	500	0/2	0,16
	2	1,000	0/2	0,0
	3	2,000	3/3	**0,0,0**
	5	4,000	3/3	**0,0,1/5**
Immunized one dose	5	2,000	1/5	**0,½**,4,4,8
	5	4,000	2/5	**½,2**,8,64,64
Immunized two doses	2	2,000	0/2	8,64
	2	4,000	0/2	32,64
	2	8,000	0/2	64,128
	2	16,000	0/2	16,128
	2	32,000	2/2	**2,4**

*Denominator represents the number of sheep injected and the numerator the number of deaths.
**Antitoxin level of sheep which died are in bold face type.

Unit of Antitoxin.—This was the smallest quantity of antitoxin necessary to neutralize completely the toxin effects of one test dose of standard toxin after an incubation of one hour at room temperature and judged by the survival of 4 of 5 mice injected intravenously and weighing 15 to 20 Gm. each.

TABLE 4—Antitoxin Titers of Immunized Lambs after Sixty-Two Days, 1947 Experiment

Immunized group	No. lots* of serums tested	No. lots showing antitoxin units/cc. serum					
		4	8	16	32	64	128
One dose	10	0	3	5	1	1	0
Two doses	10	0	0	0	9	1	0

*Each lot composed of equal quantities of serum from 10 sheep.

Combining Power (C.P.).—According to the work of Sterne and Mason,[15] combining power values are proportional to antigenicity. Such values were obtained for the toxoid contained in the bacterin used in the 1946 and 1947 experiments in the following manner: A constant quantity of product was mixed with varying quantities of antitoxin and allowed to incubate for one hour at room temperature, after which time T.D./0.14 (2^+ m.l.d.'s) of toxin was added to the mixture, and an additional incubation of one hour was allowed. The mixture was then injected intravenously into mice. For control purposes, mice were also injected with bacterin and with toxin (table 1.)

Antitoxin determinations of sheep serums collected during the experiment were made for 1/5 unit and above by using a fractional test dose. Never less than T.D./0.14 (2^+ m.l.d.'s) was used in each mouse dose of toxin and serum. Two mice, weighing between 15 and 25 Gm. each, were inoculated intravenously with each combination of serum and toxin after a neutralization period of one hour at room temperature. No attempt was made to determine over 128 units per cubic centimeter. Antitoxin levels were determined on the following serums:

On feed (days)	Controls (No.)	No. immunized		Total
		Single dose	Double dose	
32	20	20	20	60
60	10	10	10	30
62	0	100	100	200

A summary of the antitoxin content of the 32-day serums is shown in table 2.

The sheep from which the sixty-day samples were obtained were subjected to intravenous injections of varying quantities of standard toxin to determine their resistance. No attempt was made to determine the m.l.d. for sheep in the control group but rather to find a dosage of toxin certain to produce death in the majority of these animals injected. The results of this phase of the experiment are summarized in table 3.

The 62-day serums were obtained at the time of slaughter of the first sorting of the immunized group. The serum from each group of these specimens was pooled in equal quantities in lots of ten, and the antitoxin content determined (table 4).

RESULTS

Postvaccinal lameness was the only undesirable reaction observed from the injection of bacterin. The lameness was transient, lasting twenty-four to forty-eight hours in the majority of the animals. The reaction was not severe, being no greater than that following the injection of any other biologic agent of similar nature. Three sheep were lost during the immunization procedure due to asphyxia caused by overcrowding. One animal became sufficiently lame to be culled.

TABLE 5—Summary of Results of 1946 Experiment

	Control Group	Immunized Group
Total sheep	1,903	1,880
Av. days on feed	62.7	63.3
Wt. on feed (lb.)	74.81	78.31
Wt. off feed (lb.)*	103.9	106.6
Gain/day (lb.)	0.46	0.45
Feed/day (lb.)	4.14	4.04
Corn & molasses/day (lb.)	1.79	1.72
Grades (includes culls)		
choice	1,582	1,661
good	248	175
medium	20	17
cull	1	2
condemned	1	2
Dressed wt. (lb.)	47.2	48.3
Dressed yield (%)	45.4	45.3
No. culled during expt.	30	11
No. dead	51	23

*Shrinkage allowed, 4 per cent.

No blemish that could be attributed to the injection of the bacterin was observed on the carcasses of any of the immunized sheep at the time of their slaughter.

Combining power determinations of the immunizing agents used in the 1946 and 1947 experiments (table 1) show that the product used in 1947 was five times as potent (50 combining units) as that used in the 1946 experiment (10 combining units).

1946 Experiment—The results of the 1946 experiment are summarized in table 5.

The only significant differences in the control and immunized lambs were the death loss and culling loss which was, respectively, over two times and almost three times as great in the control lot as compared to the immunized lot.

1947 Experiment—The results of the 1947 experiment are summarized in table 6.

TABLE 6—Summary of Results of 1947 Experiment

	Control Group	Immunized Group
Total sheep	1,970	1,967
Av. days on feed (lb.)	71.5	69.6
Wt. on feed (lb.)	64.6	65.7
Wt. off feed (lb.)*	87.7	86.9
Gain/day (lb.)	0.32	0.31
Feed/day (lb.)	2.99	2.82
Corn & molasses/day (lb.)	1.54	1.67
Grades (includes culls)		
choice	1,720	1,763
good	131	153
medium	12	7
cull	8	9
condemned	4	4
partial salvage	17	15
Dressed wt. (lb.)	40.0	40.2
Dressed yield (%)	45.4	46.2
Culled during expt.	17	17
Deaths**	73	9

*Shrinkage allowed, 4 per cent.
**Five additional animals in each group were experimentally killed with toxin.

Of particular significance is the comparatively small loss of immunized animals. This difference was due to the decreased number of enterotoxemia losses as shown by the differential diagnostic chart, table 7. Three immunized lambs, 2 from the single dose group, and 1 from the double dose group, and 66 of the control lambs were positively diagnosed as enterotoxemia. Losses from causes not definitely diagnosed as enterotoxemia were relatively small, totaling 6 deaths and 17 culls for the immunized group and 12 deaths and 12 culls for the control group.

The average daily gain, based on live weight averages, was 0.01 higher for the control group; however, the average dressed yield of the vaccinated group was 0.8 per cent higher than that of the control group. The controls consumed an average of 1.56 lb. of corn and molasses as compared to 1.67 lb. for the immunized group. No significant differences occurred in the grading of the carcasses. Fat lambs sorted from the control and immunized groups were not weighed or slaughtered on the same dates.

The daily feed consumption, based upon weekly averages, and the losses from enterotoxemia are shown in table 8. For the first forty days the feed consumption was kept approximately at the same level for both lots. Losses from enterotoxemia in the control lot started on the twenty-fourth day. Of the 66 definite cases of enterotoxemia diagnosed in the control group, 55 occurred between the thirty-seventh and sixtieth day. During this period, the enterotoxemia loss in the immunized group was 2 animals, and the consumption of fattening elements was maintained at the same level to 0.4 lb. higher per day in a concentration of 60 to 70 per cent of the total ration.

The pathologic picture of enterotoxemia was found to correspond closely to that described by others.[1,2,5,7,12,16] The most consistent finding was the presence of cardiac hemorrhages, the size and location of which

TABLE 7—Differential Diagnostic Results, 1947 Experiment

Diagnosis	Control lot			Immunized lot		
	Cull	Dead	Total	Cull	Dead	Total
Enterotoxemia						
Toxin demonstrated	5	61	66	0	3	3
Toxin not demonstrated	1	[illegible]	[illegible]	[illegible]	1	1
Pneumonia (pasteurellosis)	6	2	8	5	1	6
Enteritis (salmonellosis)	0	1	1	3	1	4
Culls (injuries, poor condition)	5	0	5	9	0	9
Asphyxia	0	2	2	0	3	3
Undetermined	0	[illegible]	[illegible]	[illegible]	0	0
Totals	17	73	90	17	9	26

varied considerably; minute petechiae to large ecchymoses located subendocardially, subepicardially, myocardially; or any combination thereof. The complete absence of other outstanding gross pathology was not unusual. Only those cases in which the Type D toxin of *Cl. perfringens* could be demonstrated were positively diagnosed as enterotoxemia. Postmortem lesions indicated 74 deaths due to enterotoxemia, and all but 5 of these were substantiated by laboratory confirmation (table 7). None of the specimens from the 29 lambs having lesions other than those of enterotoxemia contained toxin.

The antitoxin content of serums obtained after thirty-two days on feed (table 2) was 2 or more units per cubic centimeter in all 20 lambs which had received one dose of bacterin; 8 or more units per cubic centimeter in all of 20 lambs which had received two doses of bacterin, and from 1/5 to 8 units per cubic centimeter in 5 of the 20 lambs in the control group.

The relative resistance to intravenous injections of toxin and the corresponding antitoxin titers of the control and immunized lambs after sixty days on feed is shown by table 3. Nine of the ten single dose immunized lambs contained ½ to 64 units per cubic centimeter of serum. Of these, 4 of 5 survived when injected with a quantity of toxin which destroyed all of the 3 control lambs injected; and 3 of 5 survived twice this quantity of toxin. Lambs which received two doses of bacterin showed considerably greater resistance to toxin injections and correspondingly higher antitoxin titers. Of the 10 control lambs, 2 had an antitoxin titer of 1/5 and 16 units per cubic centimeter, respectively, while no antitoxin was detected in the remaining 8 lambs.

TABLE 8—A Comparison of the Average Daily Consumption of Corn and Molasses and Enterotoxemia Losses, 1947 Experiment

Week ending	Corn and molasses consumed/day*		Enterotoxemia loss	
	Controls	Immunized	Controls	Immunized
Oct. 11.....	0.62 lbs.	0.61 lbs.	0	0
Oct. 18.....	0.99 lbs.	0.94 lbs.	0	0
Oct. 25.....	1.39 lbs.	1.29 lbs.	0	0
Nov. 1.....	1.75 lbs.	1.70 lbs.	0	0
Nov. 8.....	1.65 lbs.	1.62 lbs.	4	1
Nov. 15.....	1.95 lbs.	1.86 lbs.	4	0
Nov. 22.....	1.61 lbs.	1.77 lbs.	29	1
Nov. 29.....	1.71 lbs.	2.12 lbs.	9	0
Dec. 6.....	1.79 lbs.	2.23 lbs.	14	0
Dec. 13.....	1.76 lbs.	2.14 lbs.	2	1
Dec. 20.....	2.14 lbs.	2.29 lbs.	0	0
Dec. 27.....	1.49 lbs.	1.66 lbs.	2	0
Jan. 3.....	1.13 lbs.	1.54 lbs.	2	0
Jan. 10.....	1.25 lbs.	1.39 lbs.	0	0

*Based on weekly averages.

The antitoxin titers of the 100 serums, pooled in lots of ten and collected from each of the immunized groups of lambs at the time of their slaughter, are shown on table 4. From 8 to 64 units per cubic centimeter were present in the single dose group, and from 32 to 64 units per cubic centimeter were present in the double dose group.

DISCUSSION

The experimental data presented in this paper on the investigation conducted in 1947 leave no doubt of the ability of the bacterin to establish an active immunity to ovine enterotoxemia under feedlot conditions. A single dose of the product produces a high antitoxin response detectable in quantity sixty-two days after administration and, in all likelihood, of considerably longer duration. Two doses of the product confer an even greater protection as judged by antitoxin levels and resistance to injections of toxin. Under normal feeding conditions of approximately three months duration, one dose of the bacterin in all likelihood provides sufficient protection for that period.

Under average feedlot conditions, an adequate period elapses for the establishment of an active immunity, because the typical western lamb requires two to three weeks before becoming adapted to the new type of feed. Thus, the consumption of fattening elements does not reach a critical level before immunity is established.

Muth[1,12] deserves the credit for first investigating the use of alum-precipitated *Cl. perfringens* toxoid in this country. His method of determining antitoxin levels,

even though capable of measuring only comparatively large amounts, revealed a relatively high antitoxin level in both single and double dose groups of lambs. Antitoxin levels determined by using the more sensitive method described herein further substantiate his findings.

The relatively low degree of protection to enterotoxemia obtained by Whitlock and Fabricant[2] using English anaculture possibly can be accounted for by the absence of alum in the product. The work of Buddle[10] with alum and nonalum-treated toxoid, as well as the work of others with tetanus and diphtheria toxoids, has clearly shown that alum enhances and prolongs the immunity obtained with these types of immunizing agents.

The presence of antitoxin in the control animals indicates that a natural resistance to enterotoxemia may be established through exposure and that apparently subclinical intoxications likely occur, although it is generally conceded by those who have worked with enterotoxemia that animals which exhibit symptoms rarely, if ever, recover. This factor is further substantiated by the observation that older sheep rarely are affected by enterotoxemia.

Contrary to the method of controlling enterotoxemia by means of feeding sulfur,[16,17] the use of *Cl. Perfringens* Type D Bacterin does not result in decreased appetite and increased cost per pound of gain.

By establishing an active immunity to enterotoxemia through the use of this biologic agent, minimal enterotoxemia losses, faster gains, and lower overhead costs, through the use of self feeders and a shortened period of handling, are all within the realm of possibilities.

SUMMARY

Experiments were conducted during the winter feeding seasons of 1946 and 1947 to determine the effectiveness of the bacterin in controlling enterotoxemia losses in feedlot lambs. The 1946 experiment was well controlled but conducted with a minimum of technical work. It yielded encouraging but nonconclusive results.

The 1947 experiment was conducted under rigidly controlled and critical laboratory conditions. Combining power values indicated that the bacterin used in this experiment was five times more potent than that used in 1946.

Upon their arrival at the feed lot, 1,967 lambs were immunized with 5 cc. of the bacterin, and 960 of these were given a second 5-cc. dose two weeks following the administration of the first. Controls were 1,970 lambs derived from the same source as the immunized group.

During the course of the feeding operation, 66 controls and 3 immunized lambs developed enterotoxemia.

Antitoxin levels determined thirty, sixty, and sixty-two days after the first immunization procedure showed that the immunized lambs of both the one- and two-dose groups were resistant to the Type D Toxin of *Clostridium perfringens*.

The daily ration of corn and molasses fed the immunized lambs was maintained at approximately the same level to 0.4 lb. higher than that fed the controls.

CONCLUSIONS

1) One dose of 5 cc. of *Clostridium perfringens* Type D bacterin (Corn States) is capable of producing a high degree of protection from ovine enterotoxemia to feedlot lambs for at least sixty-two days.

2) Lambs so immunized can be fed larger quantities of fattening elements than customarily fed, with little danger of ovine enterotoxemia losses.

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A Technique for the Isolation of Newcastle Disease Virus, Using Streptomycin as a Bacterial Inhibitor

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THE SIMILARITY of the symptoms of Newcastle disease [avian pneumoencephalitis] to those of several other poultry diseases emphasizes the desirability of isolation of the virus in making a diagnosis. Although the diagnosis of Newcastle disease by serum-neutralization tests is a widely practiced method in poultry disease laboratories, ambiguous positive results may be obtained, particularly from serum collected from older birds. It is the general consensus that birds recovered from the disease may retain significant antibody titers in their serum throughout life. Thus, a positive serum-neutralization test does not indicate whether the flock in question is currently recovering from an epizoötic of the disease or whether the antibody titer is from a more distant past infection. If the virus is actually isolated and identified immunologically, the presence of the disease is established unequivocally.

Even though the virus of Newcastle disease is readily cultivated in embryonated eggs, attempts at isolation of the virus from specimens submitted to laboratories have been discouraging because bacterial contaminants produced death in embryos before the virus could become established. Attempts to remove bacterial contaminants by bacteria-retaining filters are often disappointing in that an excessive amount of virus is absorbed by the filter. This is more understandable after a study of the virus morphology in the electron micrographs by Cunha, *et al.*[1]

The introduction of streptomycin and its effectiveness, particularly against gram-negative organisms, have led to a number of reports of its successful use, alone or in combination with penicillin, in overcoming bacterial contamination. A cursory review of the literature reveals that it has been used successfully in the isolation of psittacosis virus from urine, feces, and soil,[2] and it has prevented bacterial contamination of chicken embryos inoculated with unfiltered sputums[3] and stool.[4] With its aid, influenza virus has been isolated from lung tissue,[5] and avian bronchitis virus and Newcastle disease virus from respiratory exudates.[6] It also has been used to obtain bacteria-free cultures of *Trichomonas fetus*[7] and *Trichomonas vaginalis*,[8] and in medium for the isolation of pathogenic fungi.[9] These reports led us to investigate further the practicability of using streptomycin routinely in the examination of tissues for Newcastle disease virus.*

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*Experiments along this line have been reported by: Beaudette, F. R., Bivins, J. A., Miller, B. R., Hudson, C. B., and Black, J. J.: Studies on the Diagnosis of Newcastle Disease in New Jersey. Am. J. Vet. Res., *9*, (1947): 69-76; Brandly, C. A., Moses, H. E., Jungherr, E. L., and Jones, E. Elizabeth: The Isolation and Identification of Newcastle Disease Virus. Am. J. Vet. Res., 7, (1946): 289-306.

The purpose of this paper is to present a simple technique to thwart bacterial contamination in the isolation of Newcastle disease virus with streptomycin and to report the results obtained in a large number of tests.

MATERIALS AND METHODS

Most of the tissues examined in this experiment were collected during naturally occurring outbreaks of suspected Newcastle disease. At autopsy, the lungs were collected as an individual sample and the brain, liver, and spleen as a pooled sample. Ordinary aseptic precautions were followed in collecting the tissues at autopsy. They were immediately frozen and stored in a carbon dioxide ice cabinet until examination for Newcastle disease virus could be made, which with some samples was as much as ninety days later. When embryo inoculations were to be made, the tissues were allowed to thaw either at room temperature for several hours or overnight in the refrigerator. The brain, liver, and spleen samples were triturated in TenBroeck glass tissue grinders and mixed with several volumes of sterile nutrient broth. The lungs were ground with mortar and pestle and suspended in a similar volume of nutrient broth. The suspensions were centrifuged a few minutes to settle the tissue particles. This is an important step; bacteria which are deeply seeded in shreds of tissue are apparently shielded from the action of streptomycin and often introduce bacterial infection into the embryo. The 1.75 cc. of the supernatant fluid was placed in a sterile tube and 0.25 cc. of a 200-mg./cc. solution of streptomycin was added and mixed with it. (This is 25 mg. of streptomycin/cc. of tissue suspension.) After the mixture was allowed to stand at room temperature for thirty minutes, 4, 12-day-old embryos were inoculated *via* the allantoic cavity with 0.3 cc. of the streptomycin-treated tissue extract, ¾ in., 27-gauge needles being used. Rough, quantitative bacterial determinations were made of the tissue extract before and after treatment with streptomycin by allowing 0.1 cc. of the inoculum to spread evenly over the surface of a plain agar slant. All eggs with embryos dying after twenty-four hours were saved. The allantoamnionic fluid was harvested from them and cultured in broth for sterility and, if sterile, passed again in 3 more 12-day-old embryos. If all 3 of these embryos died on the second and third days after inoculation, the allantoamnionic fluid was harvested and cultured for sterility. The presence of Newcastle disease virus was then established by serum-neutralization tests. Emryo m.l.d. titers of 10^{-7} to 10^{-9} were obtained with each virus on this passage.

EXPERIMENTAL DATA

In the beginning, it was deemed essential to know the toxicity of streptomycin for 12-day-old embryos. With a starting dose of 10 mg. (10,000 units)/egg and an increase in dosage of 5 mg. in each series, 3 eggs were injected with each amount up to 40 mg. The eggs were observed daily for six days. All the embryos receiving the 10-mg. dose lived. One embryo in each of the 15-mg. and 20-mg. dose groups died on the fourth day, and 2 of the 3 embryos receiving the 25-mg. and 30-mg. doses died on the third day. The embryos receiving the two remaining doses all died. Using streptomycin at the rate of 25 mg./cc. of tissue extract, and inoculating each egg with 0.3 cc. of the mixture, resulted in each egg receiving about 7.5 mg. of streptomycin. This seemed significantly below the toxic dose of streptomycin for embryos.

Experiment 1.—By the procedure described, 258 samples of tissues were examined for Newcastle disease virus, and 49 recoveries were made. Also, 85 samples of yolks gathered from a laying flock during a natural outbreak of the disease, each sample made by pooling yolks from two to four eggs, were examined by the same method, and three recoveries of virus were obtained. The isolations were from eggs gathered at the beginning of the outbreak. At least 13 of the tissue samples yielding virus could be arbitrarily classified as heavily contamined with bacteria. Only five of the samples yielding virus were completely sterile. No attempt was made to identify any of the various bacterial contaminants present in the samples, because the purpose of our work was to determine the practicability of using streptomycin to defeat the bacterial contamination generally encountered in Newcastle disease virus isolation, not to determine its inhibitory effect against specific organisms. Although no bacterial growth was observed on any culture of the tissue extract after treatment with streptomycin, three streptomycin-tolerant organisms were recovered from allantoamnionic fluid of embryos inoculated with the streptomycin-treated tissue extracts. Two of these were from badly decomposed specimens submitted to us through the mails.

Experiment 2.—In view of the high cost of streptomycin, an experiment was performed to determine if lesser amounts of streptomycin would effectively eliminate bacterial contamination from tissue. Nine of the heavily contaminated samples from which virus had been recovered were selected and reëxamined for virus with 25 mg. (the original concentration of streptomycin), 12.5 mg., 6.25 mg., and 3.125 mg. of streptomycin/cc. of tissue extract. Sterile allantoamnionic fluid was recovered from all dead embryos in even the lowest con-

centration of streptomycin in all nine samples.

From these results, it may be concluded that the use of lower concentrations of streptomycin under the described conditions is practicable and would considerably reduce the cost of the procedure.

Experiment 3.—Another experiment was conducted to determine the effect of prolonged treatment of tissues with streptomycin on the viability of Newcastle disease virus at room temperature and refrigerator temperature. Four samples of tissue from which virus had been isolated were reground in TenBroeck glass tissue grinders and suspended in 10 volumes of sterile broth, so that ample amounts of material would be available for daily testing for the presence of virus. Also, a 10^{-1} dilution of Newcastle disease virus (California strain 11914) in sterile broth was held at both room temperature and refrigerator temperature and titered daily to determine the loss of virus. Twenty-five mg. of streptomycin/cc. of sample was added to both room temperature and refrigerator test materials. The tissue samples were injected daily into eggs for virus recovery. Virus was isolated at the end of twenty-four hours from all four of the tissue samples held at room temperature, from two of the samples at the end of forty-eight hours, and from only one sample at the end of four days. At the end of seven days, virus was still recoverable for all four samples held in refrigeration. The California strain 11914 had a titer of 10^{-8} at the beginning of the experiment and, after seven days at room temperature, the titer had dropped to 10^{-6}, while in the sample held in refrigeration, the titer remained 10^{-8} after seven days. We interpret this finding to mean that streptomycin has no deleterious effect on Newcastle disease virus and that the loss of virus in the samples held at room temperature is due to the detrimental effects of this temperature. This undoubtedly explains why we sometimes fail to recover virus in samples submitted without refrigeration but later do obtain positive serum-neutralization tests from the same flock. The results of this experiment also suggest that persons collecting tissue specimens under field conditions might suspend them in a solution of streptomycin for shipment to a laboratory for recovery of virus and thus protect them from bacterial decomposition.

CONCLUSIONS

The procedure described brings the technique of Newcastle disease virus isolation to a point as practicable as the growth of bacteria on artificial mediums. The technique, with few modifications, is probably adaptable to the isolation of other viruses *in vivo*, either in embryos or laboratory animals; *i.e.*, equine encephalomyelitis, rabies, vesicular stomatitis, laryngotracheitis, fowl pox, etc., and perhaps the starting of tissue cultures without bacterial interference.

SUMMARY

By using streptomycin in a concentration of 25 mg./cc. of tissue extract, it was possible to make 49 isolations of Newcastle disease virus from 258 tissue specimens. Also, with the same procedure three isolations of virus were made from 85 yolk specimens gathered during a naturally occurring outbreak of the disease in a laying flock. By this method, the virus is recovered free from contamination, and the negative result, so frequently obtained following removal of contaminants by filtration, is avoided.

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Relative Activity of Sulfamethazine and Sulfaquinoxaline Against Eimeria Tenella Infection in Young Chickens

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THE VALUE of sulfaguanidine against coccidiosis of poultry has been shown by various investigators.[1, 2, 3] Waletzky and Hughes[4] compared sulfaguanidine and sulfamethazine and, according to their method of evaluation, found that sulfamethazine was five times as active as sulfaguanidine. Levine[1] found that sulfaguanidine was effective at a level of 1 per cent in feed against *Eimeria tenella* and 1.5 per cent against *Eimeria necatrix*. Sulfamethazine was recommended at the rate of 0.4 per cent in feed by Swales.[5] Delaplane, Batchelder, and Higgins[6] reported good therapeutic activity when sulfaquinoxaline was used at the rate of 0.05 per cent in mash. Grumbles, Delaplane, and Higgins[7] also found that 0.0125 per cent sulfaquinoxaline, when given continuously in an all mash ration, gave good protection against both *E. tenella* and *E. necatrix*. The effectiveness of this small amount of sulfaquinoxaline, when compared with the other sulfa drugs, indicated the desirability of comparing the activity of sulfamethazine and sulfaquinoxaline against *E. tenella* infection.

MATERIALS AND METHODS

All birds used in these experiments were battery reared and were 3 weeks old at the beginning of each experiment. The birds in experiments 1, 2, and 3 were sexed Leghorn cockerels, while Barred Rocks and crosses of mixed sex were used in experiment 4. Each experiment was conducted in a battery brooder containing ten individual units. The 20 chicks used for each group were weighed and identified on the day of inoculation. All groups were kept for two weeks, and group weights were obtained at one- and two-week intervals.

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Dr. Grumbles has joined the Veterinary Science Department, Louisiana State University, Baton Rouge. At the time of this study, he was associated with Dr. Delaplane, professor of poultry husbandry, in the Poultry Husbandry Department, Rhode Island State College, Kingston.

The inoculum used consisted of the entire infected cecum and contents, which were collected, placed in a Waring blender with 1.5 per cent potassium dichromate solution, and agitated for about three minutes. The oöcysts were allowed to sporulate in open Petri dishes and were counted by means of a standard hemocytometer. Approximately 50,000 sporulated oöcysts per birds were used as a standard inoculum which was deposited in the crop by means of rubber tubing attached to a hypodermic syringe.

Medication was given in the mash in all instances during a four-day feeding period. The activity of the drugs being compared was measured by the amount of the drug in the mash required to give equal protection when compared with a certain concentration of the other drug. Thus, if 0.02 per cent sulfaquinoxaline and 0.08 per cent sulfamethazine gave equal results, according to this method of evalution, sulfaquinoxaline was considered four times as active as sulfamethazine. Mortality was given the most consideration in evaluating the results of any given treatment. Weights and evidence of cecal hemorrhage were also considered but were given positions of less importance.

No evidence of toxicity was encountered at any of the drug levels used, and variation in weight was attributed to the severity of the infection rather than to toxicity or unpalatability of the medicated feeds.

Experiment 1 (table 1) consisted of six groups of birds. Pen 3 began receiving 0.05 per cent sulfaquinoxaline seventy-two hours after inoculation, while pen 6 first received 0.05 per cent sulfaquinoxaline after ninety-six hours. Pen 5 was given 0.1 per cent sulfamethazine seventy-two hours after inoculation, and pen 8 received 0.1 per cent sulfamethazine after ninety-six hours. Pen 1 was the noninfected, nonmedicated control while pen 2 served as the infected, nonmedicated control.

From previous observations and the results of experiment 1, it was decided that administering the drugs seventy-two hours after inoculation would probably provide the best basis for comparison; therefore, in experiments 2, 3, and 4, all medication was started seventy-two hours after inoculation and given over a four-day period.

In experiment 2 (table 2), sulfamethazine was used at levels of 0.125, 0.15, 0.1, and 0.075

per cent. The sulfaquinoxaline levels used were 0.05, 0.045, 0.04, and 0.035 per cent.

Experiment 3 (table 3) consisted of nine groups of chicks. In this test, sulfamethazine was used at levels of 0.1, 0.08, 0.06, and 0.05 per cent, while sulfaquinoxaline was given at levels of 0.035, 0.02, and 0.0125 per cent.

In experiment 4 (table 4), two levels of each drug were used and duplicate pens were tested at each level. Sulfamethazine was given at the rates of 0.08 and 0.06 per cent, while sulfaquinoxaline levels of 0.02 and 0.0125 per cent were employed.

the first week after the birds were placed on the floor and was given continuously until the birds were 11½ weeks old. Pen 2 was a control group and received no medication.

DISCUSSION

Experiment 1 (table 1) was a preliminary test made to obtain some information on the value of each drug when given at different time intervals after inoculation. The group receiving 0.1 per cent sulfamethazine after seventy-two hours (pen 5) suffered a

TABLE 1—Experiment 1, Comparative Action of Sulfaquinoxaline and Sulfamethazine Against Eimeria Tenella Infection in Chickens

Pen	No. birds	Age (wk.)	No. oöcysts	Medication	Mortality (%)	Ave. gain, two wk. (Gm.)
1	20	3	None	None	0	135.04
2	20	3	50,000	None	85	34.40
3	20	3	50,000	S.Q. 0.05%	0	84.04
5	20	3	50,000	S.M. 0.1%	40	87.40
6	20	3	50,000	S.Q. 0.05%	55	78.37
8	20	3	50,000	S.M. 0.1%	60	55.61

S.Q. = sulfaquinoxaline; S.M. = sulfamethazine.
Medication pens 3 and 5, seventy-two hours after inoculation; medication pens 6 and 8, ninety-six hours after inoculation.

Sulfamethazine and sulfaquinoxaline were also compared in prophylactic value under field conditions. This work was conducted on a commercial broiler plant, and the conditions under which this farm operated have been described in another publication.[6] Three pens of birds on the second story of a three-story broiler house were used for this study. The birds were battery reared for three weeks before being placed on the floor. Clean litter was used at the beginning in each pen, but no cleaning was done after the birds were started. There was an interval of one week between the age and starting time of each pen. Pen 3 was started first; pen 2, one week later; and pen 1, two weeks after pen 3.

Pen 1 was given 0.0125 per cent sulfaquinoxaline in an all mash ration, while pen 3 received 0.05 per cent sulfamethazine. Medication was started in both instances during

mortality of 40 per cent, while the group receiving 0.05 per cent sulfaquinoxaline (pen 3) suffered no mortality. This would indicate that sulfaquinoxaline was more than twice as active as sulfamethazine. The pen receiving 0.1 per cent sulfamethazine after ninety-six hours (pen 8) had a mortality of 60 per cent, while those receiving 0.05 per cent sulfaquinoxaline (pen 6) after ninety-six hours had a mortality of 55 per cent. Since there was no significant difference in weights of the birds in these two pens, and with allowance for biologic variation, it can be assumed that approximately equal results were obtained. Thus it would appear that half the amount of sulfa-

TABLE 2—Experiment 2, Comparative Action of Sulfaquinoxaline and Sulfamethazine Against Eimeria Tenella Infection in Chickens

Pen	No. birds	Age (wk.)	No. oöcysts	Medication	Mortality (%)	Ave. gain, two wk. (Gm.)
1	20	3	None	None	0	118.04
2	20	3	50,000	None	80	102.15
3	20	3	50,000	S.M. 0.125%	0	88.53
4	20	3	50,000	S.Q. 0.035%	0	88.53
5	20	3	50,000	S.M. 0.15%	0	79.45
6	20	3	50,000	S.Q. 0.05%	0	90.80
7	20	3	50,000	S.M. 0.1%	20	82.30
8	20	3	50,000	S.Q. 0.045%	0	93.07
9	20	3	50,000	S.M. 0.075%	30	66.80
10	20	3	50,000	S.Q. 0.04%	0	95.34

S.Q. = sulfaquinoxaline; S.M. = sulfamethazine.
Medication in all instances was started seventy-two hours after inoculation and continued for four days.

quinoxaline is of equal value to sulfamethazine where treatment is delayed for ninety-six hours.

In the second test (table 2), the group on 0.1 per cent sulfamethazine (pen 7) suffered a 20 per cent mortality, while those on 0.035 per cent sulfaquinoxaline (pen 4) had no mortality. Since 2.86 times as much sulfamethazine as sulfaquinoxaline was used in this comparison, it would seem that sulfaquinoxaline was at least this much more active than sulfamethazine. Neither the group receiving 0.125 per cent sulfamethazine (pen 3) nor the one receiving 0.035 per cent sulfaquinoxaline (pen. 4) suffered any mortality. Also, the average gain per bird over a period of two weeks in these two groups was equal; thus, it can probably be assumed that the results obtained in this comparison were approximately equal. If this is correct, it would indicate that sulfaquinoxaline was three and five-tenths times as active as sulfamethazine.

In experiment 3 (table 3), 0.02 per cent sulfaquinoxaline (pen 4) gave better results than 0.06 per cent sulfamethazine (pen 8), as there was a 10 per cent mortality in the former and a 20 per cent mortality in the latter. This would indicate that sulfaquinoxaline was more than three times as active as sulfamethazine. Comparing 0.02 per cent sulfaquinoxaline (pen 4) and 0.08 per cent sulfamethazine (pen 7), the indications are that sulfaquinoxaline was four times as active as sulfamethazine, since these two levels gave approximately equal results. Also, 0.0125 per cent sulfaquinoxaline (pen 5) and 0.05 per cent sulfamethazine (pen 9) yielded approximately the same results. This would again suggest that sulfaquinoxaline had an activity four times that of sulfamethazine.

TABLE 3—Experiment 3, Comparative Action of Sulfaquinoxaline and Sulfamethazine Against Eimeria Tenella Infection in Chickens

Pen	No. birds	Age (wk.)	No. oöcysts	Medication	Mortality (%)	Ave. gain, two wk. (Gm.)
1	20	3	None	None	0	133.93
2	20	3	50,000	None	55	46.66
3	20	3	50,000	S.Q. 0.035%	5	109.57
4	20	3	50,000	S.Q. 0.02%	10	96.85
5	20	3	50,000	S.Q. 0.0125%	45	90.59
6	20	3	50,000	S.M. 0.1%	5	94.62
7	20	3	50,000	S.M. 0.08%	15	104.82
8	20	3	50,000	S.M. 0.06%	20	97.04
9	20	3	50,000	S.M. 0.05%	35	82.74

S.Q. = sulfaquinoxaline; S.M. = sulfamethazine.
Medication in all instances was started seventy-two hours after inoculation and continued for four days.

Experiment 4 (table 4) compared 0.08 per cent sulfamethazine (pens 4 and 8) with 0.05 per cent sulfaquinoxaline (pens 3 and 7). The results indicate that sulfaquinoxaline in this comparison was from two to four times as active as sulfamethazine. On the other hand, an average of the two pens on 0.06 per cent sulfamethazine (pens 6 and 10) and the two on 0.0125 per

TABLE 4—Experiment 4, Comparative Action of Sulfaquinoxaline and Sulfamethazine Against Eimeria Tenella Infection in Chickens

Pen	No. birds	Age (wk.)	No. oöcysts	Medication	Mortality (%)	Ave. gain, two wk. (Gm.)
1	20	3	None	None	0	104.51
2	20	3	50,000	None	79	99.52
3	20	3	50,000	S.Q. 0.02%	45	99.88
4	20	3	50,000	S.M. 0.08%	45	116.69
5	20	3	50,000	S.Q. 0.0125%	65	40.10
6	20	3	50,000	S.M. 0.06%	61	72.64
7	20	3	50,000	S.Q. 0.02%	42.1	72.94
8	20	3	50,000	S.M. 0.08%	22.2	83.34
9	20	3	50,000	S.Q. 0.0125%	40	72.64
10	20	3	50,000	S.M. 0.06%	47.3	83.23

S.Q. = sulfaquinoxaline; S.M. = sulfamethazine.
Medication in all instances was started seventy-two hours after inoculation and continued for four days.

cent sulfaquinoxaline (pens 5 and 9) shows that sulfaquinoxaline was at least four times as active as sulfamethazine.

In the field work (table 5), 0.05 per cent sulfamethazine (pen 3) was compared with 0.0125 per cent sulfaquinoxaline (pen 1). The overall mortality from coccidiosis in pen 1 was 1.7 per cent and that in pen 3 was 2.0 per cent. The overall coccidiosis mortality in the nonmedicated control group (pen 2) was 20 32 per cent. In the control group, 187 birds showed *E. necatrix* infection when autopsied, while only 4 out of the pen on 0.0125 per cent sulfaquinoxaline and 6 out of the pen on 0.05 per cent sulfamethazine showed evidence of such infection.

TABLE 5—A Comparison of the Prophylactic Value of Sulfamethazine and Sulfaquinoxaline Against Eimeria Tenella Infection in Chickens Under Field Conditions

Pen	No. birds	Starting date	Mortality			Coccidiosis (%)	Wt. 11½ wk. (lb.)
			E. tenella	*E. necatrix*	*E. tenella & necatrix* simultaneously		
1 S.Q. 0.0125%	1,591	6/ 6/47	27	4	0	1.27	1.80
2 control	1,835	5/31/47	174	98	89	20.35	1.59
3 S.M. 0.05%	1,793	5/23/47	27	4	2	2.07	2.06

S.Q. = sulfaquinoxaline; S.M. = sulfamethazine.

While the field data are limited, they indicate that 0.05 per cent sulfamethazine and 0.0125 per cent sulfaquinoxaline gave comparable results, thus supporting the laboratory findings.

This field study also indicated that 0.05 per cent sulfamethazine and 0.0125 per cent sulfaquinoxaline when used prophylactically as described were effective against *E. necatrix* as well as *E. tenella*.

CONCLUSIONS

According to the results of four experiments in which sulfamethazine and sulfaquinoxaline were used at various concentrations, sulfaquinoxaline appears to be from three to four times as effective, therapeutically, as sulfamethazine against *Eimeria tenella* when medication is started seventy-two hours after inoculation. Limited field data also indicated that only one-fourth as much sulfaquinoxaline as sulfamethazine is required for protection against avian cecal coccidiosis under field conditions when the drugs are used prophylactically.

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Sulfamerazine in the Treatment of Fowl Cholera in Turkeys

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SPONTANEOUS outbreaks of fowl cholera among turkey flocks in Illinois prompted studies to develop improved methods for prevention and control of the disease. In this paper, the results of the experimental use of sulfamerazine and its soluble sodium salt are described.

EXPERIMENTAL OBSERVATION

The strain of *Pasteurella avicida* used in this study was isolated from turkeys which had succumbed during a field outbreak of acute cholera. Following initial isolation on blood agar, subcultures grown on proteose-peptone agar containing 2 to 3 per cent horse serum produced colonies which were fluorescent in refracted light. The virulence of the organism was demonstrated by its ability to produce death consistently in turkeys within forty-eight hours after intramuscular inoculation with 5,000,000 to 250,000,000 viable organisms. Preliminary observations indicated that adult chickens were more resistant than turkeys. A number of adult chickens inoculated with doses of *P. avicida* which were lethal for turkeys lingered for several days, displaying symptoms of either encephalitis or arthritis and emaciation before they succumbed or were sacrificed.

In a preliminary observation, 40 tom turkeys of a small white variety (average weight 16 lb.) were used. These birds were separated into two groups of 30 and 10 birds each and confined in separate inside isolation quarters 15 ft. by 15 ft. The group of 30 turkeys had access to a mash containing 0.5 per cent sulfamerazine at the time they were placed in isolation quarters. This mixture was not consumed in normal amounts during a 24-hour period. The following day, to insure adequate medication, the mash containing sulfamerazine was replaced with a regular turkey mash, and sodium sulfamerazine was administered orally at a rate of 0.5 gr./lb. of body weight (71.5 mg./kg.) twice daily to each of the 30 turkeys for two days. At the time the group of 30 turkeys received the first oral dose of sodium sulfamerazine, each bird was inoculated intramuscularly with approximately 200,000,000 viable *P. avicida*. At the same time, 10 untreated control birds were exposed to *P. avicida* in the same manner. Several of the sodium sulfamerazine-treated turkeys were listless, and food consumption was reduced for a period of one or two days following exposure. No deaths occurred for five days after exposure in the group of 30 sodium sulfamerazine-treated turkeys, while the 10 untreated control birds all died within forty-eight hours. *P. avicida* was isolated from the livers and hearts of the control turkeys that died.

Fowl cholera recurred in the treated turkeys six days after the initial exposure and four days after sodium sulfamerazine treatment was discontinued. Three birds died. *P. avicida* was isolated from heart and liver tissues. Four additional oral doses (two daily for two days) of sodium sulfamerazine (71.5 mg./kg.) were given, and no losses occurred for twenty-four days, at which time the disease again appeared. Two birds died. *P. avicida* was isolated from the hearts and livers. Sulfamerazine therapy was not administered, and no additional losses occurred for sixty days, at which time the experiment was terminated.

Sulfamerazine concentrations in the blood were determined each day in 15 of the 30 treated turkeys. These turkeys were selected at random on five consecutive days after initial treatment (*see* fig. 1). The blood-sulfonamide concentration was determined according to the method of Bratton and Marshall.[1] Colorimetric readings were made with a Klett-Summerson colorimeter. The highest average sulfamerazine concentration in the blood, 16.4 mg./100 cc., was attained three hours after the fourth oral dose of sodium sulfamerazine. Levels of 1.89 to 4.97 (average 2.8) mg./100 cc. of blood were noted in different birds which had access to the sulfamerazine-mash mixture for one day. While these differences might be attributed to differences in individual consumption of the medicated mash, even more

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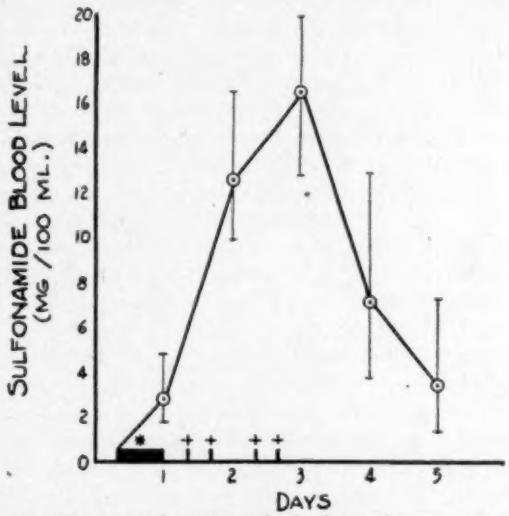
marked variations in blood-sulfonamide levels were observed when sodium sulfamerazine was administered orally at a uniform dosage rate. The second day after two oral doses of sodium sulfamerazine, the blood concentration varied from 9.97 to 16.48 (average 12.6) mg./100 cc. On the second day following the fourth oral dose of sodium sulfamerazine, the sulfonamide concentration varied in different birds from 12.85 to 19.95 (average 16.4) mg./100 cc. of blood. Twenty-four hours after therapy was discontinued, sulfonamide concentration varied from 3.08 to 12.85 (average 7.0) mg./100 cc. of blood, while at forty-eight hours the blood-sulfonamide concentration varied from 1.36 to 7.19 (average 3.4) mg./100 cc. The extremes are shown in figure 1 by the vertical lines which cross the curve of the mean blood sulfonamide level.

The result of attempted sulfamerazine therapy replaced by sodium sulfamerazine therapy in 30 tom turkeys experimentally exposed to fowl cholera indicates that this sulfonamide may prevent mortality during the period of treatment. The disease recurred three days after treatment was stopped which suggests either that the period of sulfamerazine therapy was not sufficient to destroy all *P. avicida* in 1 or more exposed birds, or that the organism may have been eliminated by body secretions or excretions before the antibacterial action of sulfamerazine was established. Reis[2] demonstrated *P. avicida* just before death in the feces of birds experimentally infected by the intramuscular or nasal routes and in the nasal mucus very early in the course of the disease. Furthermore, Reis[3] has shown that "healthy carriers" may be found among chickens which had received simultaneous injections of virulent *P. avicida* and sulfanilamide. Recurrence of fowl cholera one month after the initial exposure suggested the presence of 1 or more carrier birds, or the survival of *P. avicida* in the enclosed pens. Following outbreaks of spontaneous fowl cholera, Pritchett, Beaudette, and Hughes[4] isolated strains of *P. avicida* of the fluorescent or intermediate types from the nasal mucosa of healthy carrier birds. Nobrega and Reis[5] have stated that healthy carriers of the fluorescent type of *P. avicida* may explain the reappearance of enzoötics of fowl cholera. These workers reported that the fluorescent type may survive as long as 15 months in the nasal passages of birds.

Blood-sulfamerazine levels in 15 experimentally exposed and treated turkeys averaged 12.6, 16.4, and 7.0 mg./100 cc. of blood, respectively, during the three days in which the disease activity was probably at its peak. The critical blood sulfonamide level capable of preventing mortality was not determined; however, it appears that the aforementioned dosage and resultant sulfonamide levels were in excess of those needed to prevent death. Extremes above and below the mean blood sulfonamide levels (fig. 1) may have been due



*=The period during which the turkeys received 0.5% sulfamerazine in the mash.

+=Administration of sodium sulfamerazine orally at the rate of 0.5 gr./lb. (71.5 mg./kg.).

Fig. 1—Blood sulfonamide levels in 15 turkeys over a period of five days.

to differences in the degree of absorption of the sulfonamide from the digestive tract, or in the rate of excretion of the sulfonamide by the kidneys.

FIELD OBSERVATIONS

A natural outbreak of fowl cholera in a flock of 2,971 tom turkeys on range provided an opportunity to use sulfamerazine therapy under field conditions. The flock had access to a sulfamerazine-mash mixture (0.5%) for five consecutive days. Daily losses were computed from 8:00 a.m. one day until 8:00 a.m. the following day. Twenty-two birds died during the day in which sulfamerazine therapy was introduced; 19 birds died the second day; 6 birds died during the third day; no birds died during the fourth day. On the fourth day, 1,960 turkeys were shipped to market. Mortality in the treated flock was 51 birds (1.9%) for eight days, which included five days with sulfamerazine therapy and three days without therapy. During this interval, a mortality of 30 (50%) occurred in a group of 60 untreated control birds which were removed to a separate range preceding sulfa-

merazine therapy in the main flock. Losses in the control group presumably would have been higher if the surviving control birds had not been shipped for market purposes. Blood sulfonamide concentrations were determined in 15 birds from the main flock three days after the flock had access to mash containing sulfamerazine. The mean sulfamerazine level was 14.1 mg./100 cc. of blood, with extremes of 8.7 to 17.5 mg./100 cc. of blood.

Fowl cholera recurred in the main flock three days after sulfamerazine therapy was discontinued. The flock had access to a second course of treated mash (0.5% sulfamerazine) for seven consecutive days. Losses in the main flock which received the sulfamerazine-mash mixture for seven days and no treatment for three days were 29 birds (3.0%). In a second group of 60 control turkeys, mortality was 30 birds (50%) during the ten-day period. This control group had remained in the main flock during the first five days in which therapy was administered and seven days after therapy was discontinued, but the control group was separated from the main flock before the second course of sulfamerazine therapy was started.

No additional losses from fowl cholera occurred until the twelfth day following the second course of sulfamerazine treatment. Two days preceding a second recurrence of the disease, the flock was attacked by dogs over a 10-acre range, and 7 birds were killed before the dogs were restrained. Five severely injured birds died on the following day. Mash containing 0.5 per cent sulfamerazine was supplied for the remaining five days as indicated in figure 2. Losses ceased on the fifth day of sulfamerazine therapy. During the period of observation (37 days), the flock was moved on five occasions to new ranges. The losses in the sulfamerazine-treated flock over the period of thirty-seven days totalled 141 turkeys (11.9%). The flock was marketed after two additional recurrences of fowl cholera had been brought under control by sulfamerazine therapy.

In the field outbreak of acute fowl cholera in turkeys, sulfamerazine added to the mash at a level of 0.5 per cent markedly reduced mortality during the period of treatment and for at least two days after treatment. According to one flock manager's record of feed consumption, there was no significant difference in consumption between the 0.5 per cent sulfamerazine-mash mixture and the regular mash. Blood-sulfonamide determinations on 15 turkeys selected from the treated flock three days after the birds had access to the sulfamerazine-mash mixture revealed levels of 8.7 mg. to 17.5 mg./100 cc. of blood. Losses ceased on the fourth day of therapy. Three days following treatment, the disease reappeared. These observations suggest the possibility of sufficient sulfamerazine retention in the body tissues to suppress *P. avicida* for two days after sulfamerazine therapy. This period of time, though brief, might aid the owner of an affected flock to arrange for marketing of apparently healthy exposed birds.

Following the outbreak of fowl cholera in this flock, recurrences of the disease were noted. A number of factors may have in-

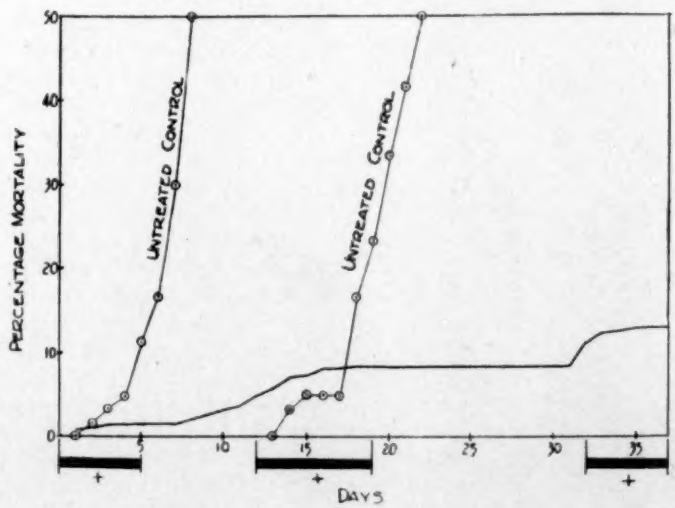


Fig. 2—Sulfamerazine therapy in an outbreak of fowl cholera in turkeys.

+ = The period during which the turkeys received therapy (0.5% sulfamerazine in the mash) is indicated by the bars.

fluenced these recurrent attacks. It appears that during an acute outbreak of cholera in turkeys all birds within a flock are rarely exposed at the same time. Moreover, a number of susceptible turkeys may have consumed sufficient sulfamerazine-mash mixture to prevent *P. avicida* from becoming established in the body. Under such conditions, the possibility of birds acquiring resistance would seem to be negligible. On the fourth day, when losses had subsided during the first course of sulfamerazine therapy in the field outbreak, 1,960 turkeys were sent to market. Excitation following handling, or moving the remaining birds to a new range, in addition to the possible presence of healthy carriers, may have been predisposing factors favoring the first recurrence of the disease. The observations of Reis and those of Nobrega and Reis, [2, 3, 5] indicate the probable importance of carrier birds in connection with the reappearance of the disease. During the second course of treatment, the flock was moved to a new range. At this time, feed and water equipment was thoroughly cleaned and disinfected with boiling lye water before moving to clean ground. These control measures, designed to destroy infective material, were followed by a reduction in the incidence of the disease. Negligible losses observed from the sixteenth until the thirty-first day are shown in figure 2. A second recurrence of fowl cholera followed an attack by dogs thirty-one days after the initial outbreak. Excessive physical stress, excitement, and injury may have lowered body resistance and activated the disease.

SUMMARY

Sulfamerazine was employed in the prevention and control of experimentally induced fowl cholera in turkeys as well as in a natural outbreak of the disease.

1) No deaths occurred among 30 turkeys exposed by intramuscular injection of *Pasteurella avicida* during the period of attempted sulfamerazine and sodium sulfamerazine treatment. In a group of 10 untreated control birds exposed in the same manner, 100 per cent mortality occurred within forty-eight hours. A recurrence of fowl cholera was noted in the treated group of turkeys, and losses stopped following four oral doses of sodium sulfamerazine over a two day interval.

2) In a natural field outbreak of fowl cholera, 0.5 per cent sulfamerazine in the mash promptly reduced mortality during the period of treatment and for two days following treatment. A mortality of 50 per cent was experienced in two groups of 60 untreated control birds over a period of eight and ten days, whereas mortality in the treated flock was 1.9 per cent and 3.0 per cent, respectively, for the same period. Four recurrences of fowl cholera were observed in the same flock following the initial outbreak, and each time losses ceased when 0.5 per cent sulfamerazine was added to the mash.

3) Blood sulfonamide concentrations in 15 experimentally treated turkeys reached the highest level (average 16.4 mg./100 cc.) within three hours after the fourth oral dose of sodium sulfamerazine (12-hour intervals, 0.5 gr./lb.). In the field trials, blood samples obtained from 15 turkeys after the treated flock had access to a 0.5 per cent sulfamerazine-mash mixture for three days revealed sulfonamide levels varying from 8.7 to 17.5 mg./100 cc. of blood.

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The Isolation and Identification of Fowl Plague Virus

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FOWL PLAGUE is one of the most fatal infectious diseases affecting poultry, particularly chickens. It has occurred in practically all parts of the world where poultry is raised. The natural disease has been present on the North American continent on two occasions, both restricted to the continental United States, in 1924-1925 and in 1929. Vigorous measures led to its eradication on each occasion. The potential threat of this destructive malady to important sources of food during circumstances of war prompted investigations pertaining to its recognition and control.[1]

Although fowl plague has long been known as a specific disease of poultry caused by a filterable virus, the degree of characterization of the disease or of the causal agent has not been such as to preclude confusion in its recognition. The necessity of differentiating the disease from fowl cholera has been uniformly acknowledged, and such differentiation was rather easily attained.[2] However, the need for further study on means of the diagnosis of the disease was emphasized by the relatively recent recognition of atypical or less acute forms of fowl plague,[3] of the nature and distribution of Newcastle disease[4, 5] and, not withstanding other evidence to the contrary,[4] by the report of Manninger on the immunologic identity of fowl plague with Newcastle disease.[6]

The report describes studies on the recovery of the causal virus from experimental cases of fowl plague, on means of the identification of the virus, and on other features of the virus that are of possible usefulness in the diagnosis of the disease. The literature having a direct bearing on the various phases of the problem is cited in subsequent sections, and the information contained in the older reports is recognized largely as presented in the critical review made by Lagrange.[3] Extended considerations of pathology[7] and immunology[8] are presented in other reports.

ISOLATION OF THE VIRUS

Origin of the Virus.—A strain of fowl plague virus, originally isolated from an outbreak of the natural disease in chickens in the Dutch East Indies, was provided for the investigation. This strain of virus was obtained from the Ministry of Agriculture and Fisheries, Weybridge, Surrey, England, where it had been passaged serially in chickens at intervals of about three months, and otherwise maintained by storage of chicken tissues in 50 per cent glycerol-saline under ordinary refrigeration. Bits of infected liver and spleen collected from an infected chicken and placed in the glycerol preservative in May, 1943, were transported by airplane to our laboratory, where attempts at cultivation of the virus were begun in July, 1943.

In addition to the Dutch East Indies (D. E.I.) strain of virus, which according to our data is a classical one, five variant viruses of this strain became available for comparative study. These variants were isolated, during the course of the laboratory investigation, from the D.E.I. strain, as indicated in subsequent sections.

Demonstration of Virus in Chicken Tissues by Inoculation of Chickens.—In the earlier investigations,[3] it was established that in typical fowl plague the causal virus attains a high concentration in the blood, or such a concentration that 0.000,001 cc. was sufficient to cause a lethal infection. The virus persisted in the blood unto death, after which, it was demonstrable in all tissues, secretions, and other body fluids. The only major irregularity in the demonstrability of the virus occurred in studies of liver tissue; "autosterilizing processes" were given extensive consideration in this regard.

This work was done as a part of a research project conducted under the direction of a War Department Commission consisting of: Brigadier General R. A. Kelser, U. S. Army; Dr. R. E. Dyer, U. S. Public Health Service; Dr. H. W. Schoening, Pathological Division, Bureau of Animal Industry, U. S. Department of Agriculture; and Dr. E. B. Fred, University of Wisconsin.

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In studies on the distribution and fate of the virus in chickens, Hallauer[9] observed that virus administered intravenously following active immunization was demonstrable in the peripheral blood for from two to four hours, but only in conjunction with the leucocytes, and in the liver and spleen up to twelve hours following injection. This worker found also, in connection with active-passive immunity (simultaneous injection intravenously of large amounts of antiserum and small amounts of virus), that again an affinity existed between the virus and the leucocytes, that, following the disappearance of the virus from this component of the blood at three hours postinjection, it reappears, even with the concurrent existence of antibodies during the symptomatic period of disease from which the bird recovered. During the period of clinical disease, the virus was recoverable also from the liver, spleen, kidney, and brain.

In our studies, the embryonating chicken egg was used almost exclusively for evaluations of the presence and distribution of virus in chicken tissues, as described in the subsequent sections. The virus contained in the splenic tissues received from England was shown to produce the rapidly fatal, classical infection when injected intramuscularly or intravenously into chickens.

An evaluation was made of the effect of the route of inoculation of adult chickens on the infectivity and lethality of the virus. The infectivity titer of the inoculum was determined also for 12-day embryonating eggs, thus providing for the expression of the quantity of the inoculum in terms of embryo minimal lethal doses (m.l.d.'s). The virus employed was contained in the serum, or in tenfold dilutions thereof, in 1 per cent peptone broth, from a chicken in the later stages of the clinical disease. The m.l.d. for chickens was essentially the same by intravenous, intramuscular, intraperitoneal, intracranial, and subcutaneous routes, or such that an m.l.d. for groups of 2 chickens was similar to that for embryos. Larger amounts of virus, possibly ten times as much, were required to produce the disease by the intradermal, oral, and intratracheal routes, and in excess of at least 1,000 embryo m.l.d.'s was required to cause lethal infection following instillation of the virus in 0.1-cc. amounts into the conjunctival sac. Of 41 chickens that died of the disease following the various exposures, the earliest deaths in the majority of them occurred about forty hours postinfection. Accordingly, it appeared that the m.l.d. for chickens and for embryonating eggs was the same, providing the virus was injected beyond the outer epithelial barriers of the chicken.

Data obtained in the course of other studies[8] revealed uniform susceptibility of male and female chickens, and of young males, irrespective of treatment with diethylstilbesterol.

Demonstration of Virus in Chicken Tissues by Inoculation of Embryonating Chicken Eggs.—The embryonating chicken egg was shown by Joaun and Staub, in 1920, to be a satisfactory medium for the propagation of the fowl plague virus.[10] The method was later applied successfully to this virus by Burnet and Ferry,[11] Burnet,[12] Ambrosioni,[13] Lush,[14] and Traub.[15] The procedure had also been used by Kimura and Masunaga,[16] although the data presented by these workers appear to be atypical for a classical strain of the fowl plague virus.

The infection produced by the plague virus terminated in death of the embryos. Congestive and hemorrhagic lesions, particularly in the skin and musculature, were considered characteristic of the infection. The virus was found to attain such a concentration in the chorioallantoic membrane (ca.m.) that 0.03 cc. of a dilution approximating 10^{-7} was infective.[11] The virus was found to be present in similarly high concentrations throughout the embryo,[12] and possibly also in the allantoic fluid.[14]

Isolation of Strain D.E.I. Virus.—The establishment of infection in embryonating eggs by this virus was quite readily accomplished. The supernate of a suspension (1:5) of the original splenic tissue in buffered broth was used in 0.03- to 0.25-cc. amounts to inoculate groups of 12-day embryonating eggs by several routes, namely, into the yolk, the allantoic or the amnionic chamber, or intracranially into the embryo itself. All 6 embryos in each group died within forty-six hours, and lesions suggestive of infection by fowl plague virus were evident. The allantoamnionic (a.a.) fluids of the eggs inoculated by the allantoic chamber (a.c.) route were found to contain high titers of virus; 0.05-cc. quantities of 10^{-7} and lower dilutions, inoculated into the allantoic chamber of 12-day embryonated eggs, were lethal within the period previously cited, and the embryos showed characteristic lesions. In this manner, usually employing the a.a. fluids of eggs inoculated with the 10^{-6} dilution, 35 serial passages of the virus were conducted. The concentration of the virus in the a.a. fluids remained quite constant throughout the passages, or usually 20×10^{6} m.l.d.'s per cubic centimeter.

The virus in the imported spleen was also isolated in embryonating eggs following a preliminary passage in chickens. Young adult White Rock chickens were infected by intraperitoneal injection of a supernate of the spleen emulsion, and, approximately twenty-four hours later and after the onset of clinical disease, blood was withdrawn from them. Serum in 0.05-cc. amounts was injected intra-

venously (allantoic vein) into 15-day embryonated eggs. When disease was indicated by pallor, sluggishness, or death in some of the embryos, after about twenty-one hours, blood was withdrawn from the allantoic veins. Coagulation of the blood was prevented by the addition of heparin. Thirty subsequent intravenous passages were conducted with this virus. The nature of the tissue changes in the embryo was similar to those in embryos of the allantoic chamber series or in embryos inoculated by the other routes, and the concentrations of virus attained in the blood, embryos, and extra-embryonic fluids were comparable to that (20×10^{-8}) obtained following a.c. inoculation.

Evaluations of routes of inoculation and age of embryos, for purposes of isolation of the virus, provided information largely on limitations of technique. The procedures of inoculation were most readily performed by using eggs incubated beyond eight days for a.c. inoculation, eight to twelve days for ca.m. inoculation, and twelve days or more for intravenous inoculation. The infection was established only irregularly by a.c. inoculation of eighteen-day embryonated eggs. This may have been related to the drop in pH that occurs in the later stages of incubation; a value of pH 6, as observed by Romanoff and Hayward,[17] was confirmed by us. Furthermore, physiologic alterations in vascularity of the extra-embryonic membranes may reduce their susceptibility. The infection produced by intravenous inoculation caused death within one-half to three-fourths of the time required by that of the other two routes.

One study was conducted on the elimination of the fowl plague virus from, and its distribution in, the bodies of infected, fully susceptible chickens.[18] Each of 2 adult White Leghorn hens was infected intramuscularly with 100 m.l.d.'s of virus, and, at three-hour intervals thereafter, the following specimens were collected: 2 cc. of blood from peripheral veins, a fecal sample, and nasopharyngeal mucus. After death, a variety of tissues and other materials was collected. The mucus (in 2-cc. broth), the feces, and the materials collected postmortem were stored for several weeks in a CO_2 refrigerator (–70 C.) except that, through an inadvertency, the specimens obtained postmortem were placed for a week at 6 C. The blood components (citrated, 0.5%) were separated and stored at 6 C. for ten days. The materials contaminated with bacteria were exposed to final concentrations of 10,000 Oxford units (O.U.) per cubic centimeter of penicillin sodium at 30 C. for one hour prior to the initiation of tests for virus. Evaluations of virus content were made by inoculation of embryonating eggs by the a.c. route with the undiluted or diluted specimens.

The results are recorded in table 1. The virus was demonstrated first (at fifteen to eighteen hours postinoculation) in the cellular components of the blood in both chickens. The acellular portions of the blood contained minimal amounts of virus six hours later, or twenty-one and twenty-four hours following injection of the respective chickens. At about this time, the virus also became demonstrable in the nasopharyngeal mucus. During the next three-hour interval, demonstrable virus was first shed in the intestinal excreta. With the exception of the thirty-hour specimen of nasopharyngeal mucus, the virus was demonstrable throughout the further course of the disease. Mild malaise was first noticed in chicken 1 fifteen hours postexposure and in chicken 2 twenty-four hours after inoculation; death occurred after thirty-five and thirty-seven hours, respectively. Of the naterials collected postmortem, the brain contained the largest amount of virus, or 2×10^7 m.l.d.'s per gram. The only other tissue in which virus was demonstrated from both chickens was lung, where the concentration of virus was one hundred fold less than in the brain. Test inoculums of the liver, pancreas, bile, crop, and duodenum of both chickens were innocuous for embryonating eggs. The demonstrability of the virus in the other materials of the 2 chickens was not uniform. Virus was found in the spleen and kidney, in the washings of the treachea and proventriculus, and in the contents of the ileum of only 1 chicken. Also, in single tests, virus was shown to be present in the yolk mass of a sessile ovum and in yolk and albumen of a fully formed egg found in the oviduct, and to be absent in the contents of the gizzard and in the tibial bone marrow.

Since the ability to demonstrate virus in the hepatic tissues was at variance with other isolated tests, and since attention had been given by others to "autosterilizing processes" in the liver, particularly as a means for preparation of vaccines, a test was undertaken to determine the influnce of storage temperature on the viability of the virus. The results showed that in a liver with a virus content of $10^{6.5}$ m.l.d.'s per gram immediately after death, the virus titer remained unchanged after a three-day interval of storage at –70 C., while at 6 C., the titer was reduced to approximately 50 m.l.d.'s per gram. An emulsion of the same liver in a 1:5 dilution of 0.85 per cent sodium chloride solution retained the original virus titer after eight days at –70 C. and showed a reduction in virus to $10^{3.5}$ m.l.d.'s per gram at 6 C. and a disappearance of virus at 37 C.

This demonstrability of the virus in tissues and other materials of fully susceptible chickens was in sharp contrast to the failure to detect the virus in chickens infected following vaccinal procedures[3] that rendered the

chickens relatively resistant to the infection. Seven clinically affected chickens, whose serums in 0.05-cc. amounts neutralized 10^6 to 10^8 m.l.d.'s of virus one week following intravenous exposure to the living virus, were used in the study.[18] The virus was demonstrated neither in the washed blood cells collected seven days postinfection nor in the feces obtained daily thereafter from 3 chickens that died eight, eleven, and eleven days postinfection and from four daily samples from 1 of the 3 chickens that recovered, nor in brain, lung, spleen, liver, testes, kidney, and intestinal washings of four chickens that died seven, eight, eleven, and eleven days postinfection.

ISOLATION OF VARIANT STRAINS OF THE D.E.I. VIRUS

Five variant strains of virus were isolated from the original D.E.I. virus. Three of the strains appeared in the course of studies with D.E.I. virus that had been propagated solely in embryonating eggs in our laboratory.[8] The other two strains originated from serum of infected, fully susceptible chickens in which classical fowl plague had been produced by injection of egg-propagated virus. In 1 instance, the virus had undergone one passage in eggs by a.c. inoculation and, in the other, 11 intravenous passages in eggs. In each instance, appearance of the variants occurred in 1 of 3 or 4 embryonating eggs inoculated with material diluted to the end point of infectivity of the parent virus. Numerous other similar titrations of aliquot portions of the source materials stored for shorter and longer periods at –70 C. revealed the presence only of the D.E.I. virus. The chicken serums that served as the source of the two strains contained 20 x 10^5 m.l.d.'s of D.E.I. virus per cubic centimeter.

The conditions that served for the recognition of the first (strain 4395) of these atypical viruses,[19] were uniform for all. The course of the infection in the embryos that yielded the variants was two to three times longer (103 and 168 hours) than the average survival time of embryos infected with the comparable dilutions of parent virus. The altered gross appearance of the spleen and little or no evidence of hemorrhages in the muscles suggested an atypical infection.[7]

Inoculations of the triturated spleens to other embryonating eggs reproduced the atypical infection. Data reported in a subsequent section and in another report[9] showed an immunologic relationship of the variants to the D.E.I. virus.

The nature of the variants appeared to remain constant on serial passage in 10- to 12-day embryonated eggs. The embryos and the a.a. fluids in eggs inoculated by the a.c. route were found to contain smaller amounts of virus than obtained with the parent virus as determined by end point lethality; the infectivity titer as shown by a.c. inoculation of eggs, was usually 20 x 10^4 m.l.d.'s of virus per cubic centimeter. The 4395 variant was used in 15 serial a.c. passages, and the other strain isolated from chicken serum underwent five such passages.

The results of assessments of routes of inoculation and ages of embryos for use in isolation of the variants were somewhat at variance with those obtained for the parent virus. Intravenous inoculation of embryos usually detected one hundredfold greater amounts of

TABLE I.—Demonstrability of Fowl Plague Virus in Materials from Chickens Affected with Experimental Disease

Chicken No.	Material tested	Antemortem: Hours postinjection* 15	18	21	24	27	30	33	36	Postmortem** Brain	Lung	Spleen	Kidney	Ovary	Sessile ovum	Oviduct egg: Yolk	Albumen	Washings from: Trachea	Proventriculus	Ileum
1	Blood cells	—	2	2	2	2	3	8												
	Plasma	—	—	—	0.1	0.1	0.1	8												
	Mucus of nasopharynx	—	—	—	+	+	—	+		7	5	5	5	—		3	3	+	+	—
	Feces	—	—	—	—	3	5	4												
2	Blood cells	0.5	2	2	3	4	5	0.5	6											
	Plasma	—	—	0.1	0.1	2	2	4	4											
	Mucus of nasopharynx	—	+	+	+	+	+	+	+	7	5	—	—	4	6			—	—	+
	Feces	—	—	—	0.5	0.5	2	4	2											

*— = virus not demonstrated; + = virus present, but titer not determined; blank space = test not conducted; and numerals indicate reciprocal of log of the titer of virus that was demonstrated.
**Symbols showing results are same as in above footnote; in addition to the results shown, virus was not demonstrated in liver, pancreas, tibial bone marrow, bile, and washings from crop, gizzard, or duodenum.

virus than was possible by either a.c. or ca.m. inoculation. The rate of development of virus and the course of the disease in 10-day embryonated eggs was more rapid than in 13-day eggs, following a.c. inoculation with 100 m.l.d.'s of virus. Similar exposure of 16-day embryonating eggs was apparently associated with death of the virus.[8]

The low virulence of the variant viruses[8] and the immunity that was demonstrable soon after their injection appeared to preclude their recovery from chicken tissues. In the earlier studies of the 4395 variant, pairs of 3-month-old chickens were inoculated intravenously with from 2 to 20 x 10^3 embryo m.l.d.'s of the virus, in amounts varying by tenfold, and 4 each received 20 x 10^4 and 20 x 10^5 m.l.d.'s. Three of the chickens that received the largest amount of virus died after forty, ninety, and 100 hours, and 1 that received 20 x 10^2 m.l.d.'s died after ninety hours. Ten-day embryonated eggs were inoculated by the a.c. route with the supernate of emulsions of the brains and of the spleens of the 4 chickens. The virus was recovered from the 3 chickens that died after the shorter periods, and it was present more regularly and in greater concentrations in the spleen than in the brain.[20] Other attempts to isolate the virus from the serum of chickens exposed intravenously to 10^2 embryo m.l.d.'s of the virus two to fourteen days previously met with failure.[20, 21]

IDENTIFICATION OF THE VIRUS

Size and Filterability.—Elford and Todd,[22] and later Burnet and Ferry,[11] determined by use of Elford, graded collodion membranes that the size of the fowl plague virus was within the range of 60 to 90mμ.

Our use of filtration procedures on materials infected with the plague virus and the several variants isolated from it was confined to Seitz filters, through which the viruses appeared to pass readily.

Effect of Penicillin.—The exposure of minimal infective doses of the D.E.I. virus and of one of the variants (4395) to amounts of penicillin sodium ranging up to 10,000 O. U. per cubic centimeter, at about 25 to 30 C., for one hour, was without effect on either the infectivity titer of the virus material or the course of the infection in embryonating eggs.[23] The eggs were inoculated by the a.c route with 0.1-cc. inoculums. The antibiotic itself, in the concentration mentioned, was without demonstrable effect on the embryo.

Following the demonstration that penicillin would adequately destroy and/or suppress bacterial interference in the isolation of Newcastle disease virus from chicken feces and other contaminated materials,[24] the procedure was successfully applied to the isolation of the fowl plague virus as described in a preceding section.

Tenacity To Heat.—One-cc. amounts of transparent a.a. fluids of lethally infected embryonating eggs were placed in vials of 2-cc. capacity, and the vials sealed and immersed in a thermostatically controlled water bath. Two vials were removed at the end of each test interval and their contents pooled and tested for infectivity. These procedures revealed that infectivity was destroyed by exposure to 60 C. for a period of five minutes or less, while at 55 C. infective virus was still present after fifteen minutes. These results agreed essentially with those obtained by earlier workers using exudates and tissues of infected chickens as the test material and the chicken as the test organism.[3]

Tenacity to Various Conditions of Storage.—The known greater preservability at temperatures of approximately –70 C. of a variety of other viruses prompted our initial use of such refrigeration as provided by a dry ice (CO_2) cabinet, and, as its efficiency soon became evident, continued and extensive use was made of it in these studies. Lyophilization of virus specimens was later employed as an adjunct, and comparative studies of the storage effect of crude and dried material at various temperatures were conducted.

The comparative studies revealed that infective D.E.I. virus materials retained their titers of infectivity best when stored in the crude form and at –70 C. This is shown in part by the data compiled in table 2. In this study, the flame-sealed vials of serum from 2 infected hens were stored in the crude and lyophilized form at –70 C., at 6 C., and at 37 C. Infective virus had disappeared in both the crude and the dried serum within eighty-five days at 37 C. At 6 C., the dried material first showed loss in titer with the virus not being demonstrable after ninety-two days of storage, while the crude material showed a loss of 99 per cent after storage for sixty-six and ninety-two days. The virus in the serum frozen at –70 C. was apparently unaltered after 242 days, the longest test period employed, and that in the dried serum showed a reduction of 1 to 10 per cent of the originally demonstrable titer. The results obtained with the dried material may have been due in part to the process of lyophilization itself or to factors involved in reconstitution of the serums, since it was observed in other tests that assessments of the virus titer of serums made immediately after lyophilization often showed a drop in titer to the next lower tenfold dilutions.

Reference was made in a previous section to the deleterious effect of storage on infected liver tissue at 6 C. and 37 C.

Other observations included numerous titrations of infected a.a. fluids stored for

periods as long as twenty-five months, but commonly for periods of two to three months at −70 C., without perceptible loss of virus titer. This observation held true even for small quantities (1 to 2 cc.), stored either in cotton-stoppered tubes or flame-sealed vials. Alterations in the nature of the infection produced in embryos or in chickens were observed neither in materials which maintained their original titer of virus nor in those that were reduced in titer under other conditions, except as described in connection with the isolation of the variant strains.

Tenacity to Ultraviolet Light.—Two sources of ultraviolet light were employed in these studies: one was a Hanovia coil emitting rays of approximately 2,537 Å wave length; the other was the apparatus of Oppenheimer, Levinson, and associates,[28] emitting rays of 1,600 to 1,800 Å, the facilities of which were provided by the National Institute of Health, Bethesda, Maryland. The rays of the Hanovia coil required twenty to forty-five minutes to inactivate the D.E.I. virus in 35 to 40 cc. of clear a.a. fluid from lethally infected eggs. The light of the apparatus emitting the shorter rays destroyed the infectivity of the virus in one and one-tenth seconds in one trial and in three seconds in another. Details of these studies are presented elsewhere.[8]

Evaluations of the effect of ultraviolet light on the hemagglutinin of the virus showed that the light associated with the shorter rays caused no alteration in the hemagglutinin titer, while that of the longer rays caused a slight reduction. The time intervals involved exceeded the exposure period that was required to destroy the infectivity by one second for the shorter rays and by approximately ten minutes for the longer rays.

Tenacity to Various Chemical Agents.—The tenacity of the fowl plague virus in the presence of nine chemicals was determined with variable degrees of detail. In all cases, the virus employed was contained in clear, nonflocculent a.a. fluids of infected eggs, and tests for infectivity were made by inoculation of embryonating eggs. The inoculums contained either 0.05 or 0.1 cc. of the chemicals in all cases; the injection of the chemical agent alone in the dilutions used was without demonstrable effect on embryonating eggs. In table 3, the results are shown of the effect of several agents at room temperature. These were in the order of lethality for the virus and from best to poorest: mercuric chloride, phenolor, cresol, clorox, potassium permanganate, and lye.* Determination of the pH of the chemical-virus mixtures showed that the effect of the lye alone could be attributed to this factor.

Studies reported in subsequent sections or elsewhere[8] provided additional information. Phenol and merthiolate,* mixed first with chicken serum in 1.0 and 0.02 per cent concentrations, respectively, and later with virus material and held at room temperature for one hour, were without effect on the virus. The virus was readily inactivated by dilute hydrochloric acid and was relatively much more resistant to sodium hydroxide under the conditions given in the next section. Virus material containing 0.4 or 0.2 per cent concentration of formalin was still active after five minutes but not after six hours; 0.1 per cent formalin inactivated the virus between eighteen and twenty-four hours; and 0.05 per cent did so in more than thirty-one hours but less than 216 hours. The variant strains of the D.E.I. virus were inactivated in a manner apparently comparable to that of the D.E.I. virus by the concentration of formalin commonly used in the production of vaccines.[8]

Tenacity to Various pH Values.—The details of studies on the pH-stability patterns of the D.E.I. virus and the 4395 variant are described in another report.[26] The results obtained with the D.E.I. virus were confirmatory to those obtained by Pyl[27] for the Brescia and Bologna strains under somewhat different test conditions. At 6 C., the D.E.I. virus survived at the maximal titer after one week within the range of pH 6 to 11; however, the stability pattern differed after exposure for only one hour, in that infectivity was still demonstrable at pH 5 and pH 12. The pattern for the variant virus was essentially similar to that of the parent virus.

Conditions of pH unfavorable for the maintenance of infectivity of the D.E.I. virus were apparently deleterious also for its hemagglutinative action. Formalin-inactivated virus in embryo tissues was nonhemagglutinative after exposure for three weeks to pH 5, while the hemagglutinin titer remained unchanged at pH 6, 7, 8, and 9.

Hemagglutinative Activity.—Lush[14] found that the a.a. fluids of embryonating chicken eggs infected by the a.c. route with a strain of the plague virus contained a hemagglutinin for chicken erythrocytes. A modification of the Hirst test[25] was employed, wherein the results were determined by the pattern of the sedimented cells. Lush observed also that this activity of the plague virus was abolished by mixing it with specific antiserum.

Our preliminary studies[29] of the activity of the D.E.I. virus were conducted by Lush's pro-

*Phenolor is a mixture of 13 per cent orthophenyl phenol, 5 per cent isopropyl alcohol, and 13 per cent soap and is made by E. R. Squibb and Sons, New York. Cresol or cresol compound, technical, was reported by its distributor to be a mixture of cresylic acids prepared by a widely used standard formula. Clorox has as its active ingredient 5.25 per cent sodium hypochlorite according to its maker, Clorox Chemical Company, Oakland, California. Lye contained 96 per cent sodium hydroxide and 0.5 per cent sodium carbonate as active agents according to its maker, the Buckeye Soda Company, Painesville, Ohio.

*Merthiolate, sodium ethylmercuri-thiosalicylate, was obtained in aqueous solution.

cedure and by a slight modification of it, in which the test was held at 25 to 30 C. instead of 6 C. and read at three subsequent fifteen-minute intervals instead of after two hours. The authors' results confirmed those of Lush on the activity of the virus for chicken erythrocytes. Allantoamnionic fluids of infected eggs were used for the most part in our studies; limited observations revealed no significant difference between the activity of this material and of the fluid portion of triturated embryos.

Our experiments were extended to include erythrocytes of other species for comparative purposes with the virus of Newcastle disease and that of influenza A (strain PR8).[21] The plague virus was found to have the most versatile hemagglutinin of the several strains of virus that were studied. Agglutination occurred with all erythrocytes tested, including those of numerous chickens and chicken embryos, 3 turkeys, 1 each of man, Muscovy duck, horse, ox, sheep, guinea pig, monkey (*Macaca rhesus*), dog, and cat, several rabbits, and 6 swine. The cells of rabbits and swine revealed end points of agglutinin titers of only one-fourth to one-eighth, respectively, of the 1:320 titer commonly observed with chicken and other erythrocytes on comparable sources of virus. The occurrence of spontaneous agglutinability of the rabbit and swine cells used by us was satisfactorily overcome for purposes of the test by the substitution of 0.25 cc. of a 1:10 dilution of homologous serum in 0.85 per cent solution of sodium chloride for a like amount of the salt solution ordinarily employed. No differences were found in comparative tests using erythrocytes from chickens that were fully susceptible and those rendered relatively immune to the specific virus

The virus agglutinin for chicken erythrocytes was also demonstrable by a test employing a suspension of cells in from 8 to 10 per cent concentration and readable within a minute or less.[20]

An absence of agglutinative ability was demonstrated for serums of chickens infected with the D.E.I. virus and for a.a. fluids and extracts of embryos infected with the variant strains of the D.E.I. virus. The embryo-infectivity titers of these materials approximated 20 x 10^8 m.l.d.'s per cubic centimeter.

The final concentrations of hemagglutinins in the a.a. fluids of embryos lethally infected by the D.E.I. virus were unaltered by postinfection incubation of the eggs at 34 C. and at 40 C., as compared at 37 C. Observations were made during nine concurrent serial passages of the virus in eggs incubated at the several temperatures. The eggs were inoculated by the a.c. route with 10 to 100 m.l.d.'s of virus.

Blockade of Hemagglutinin Action.—Hirst[20] reported that chicken erythrocytes which had adsorbed and eluted an influenza virus were rendered insensitive to the agglutinin of the homologous virus. Likewise, Burnet[22] stated that a somewhat similar procedure was found applicable to other viruses and their differentiation. Comparable *in vitro* procedures employing the D.E.I. fowl plague virus and the Hertfordshire strain of Newcastle disease virus met with failure in our hands. However, the erythrocytes of infected embryonating eggs were found to be altered in their sensitivity to the agglutinin of homologous and heterologous virus materials.

The *in vivo* studies of blockade of hemagglutination were conducted with D.E.I. plague virus, the Hertfordshire, and three California strains of Newcastle disease virus, and the PR8 strain of influenza A virus.[23] Chicken eggs in the fifteenth day of embryonation were infected intravenously with the respective viruses. The eggs were exsanguinated sixteen to twenty-four hours later, or when experience and control tests indicated that near maximal titers of the virus were obtainable; the reliability of the influenza virus, however, was unknown in this respect. A small amount

TABLE 2.—Effect of Physical State and Temperature on the Maintenance of the Infectivity of the Fowl Plague Virus in Chicken Plasma

Storage time (days)	Physical state	Storage temperature of material and infectivity titer for embryonating eggs*		
		—70 C.	6 C.	37 C.**
1	Crude		10^{-5}	
66	Crude	10^{-5}	10^{-5}	$<10^{-3}$
	Lyophilized	10^{-5}	$<10^{-3}$	10^{-3}
85	Crude	10^{-5}	10^{-4}	0
	Lyophilized	$<10^{-4}$	10^{-1}	0
92	Crude	10^{-5}	10^{-3}	0
	Lyophilized	10^{-3}	0	0
242	Crude	10^{-6}		

*Blank space = not tested; < sign = less virus present than titer that is stated.
**Hydration of the lyophilized specimens resulted in the formation of a gelatinous mass, particles of which were visible in the 10^{-3} dilution.

of heparin was used to prevent coagulation of the blood. Allantoamnionic fluids were also collected from the various groups of eggs. The blood plasma and cells were separated and the cells washed three times in saline approximately 50 times their volume, and a final 1 per cent suspension was made. The various viruses in the samples of a.a. fluids and of plasma were tested for hemagglutinative activity by using the various groups of infected cells, as well as cells from normal embryos of similar age and from normal chickens. The results showed principally that (a) the red cells infected by the plague virus were agglutinated neither by materials containing the homologous virus nor by the heterologous viruses; (b) the red cells infected with the Newcastle virus strains were insensitive to the materials containing the same or different strains of that virus, but, in comparison to the normal cells, were fully sensitive to the plague agglutinin and somewhat less sensitive to the influenza agglutinin. The influenza-infected cells were sensitive to the homologous and heterologous agglutinins; the plasma from these infected cells was without hemagglutinative activity, yet the a.a. fluids from the same eggs were active.

Plague-infected erythrocytes that were obtained in the afore-mentioned manner were agglutinated by antiserum prepared for the D.E.I. strain and for one (19493) of the variants. Only high titer serums, either undiluted or in low dilutions, were assessed for this property.

Mouse Infectivity.—While both failure and success are recorded in reports on studies of the infectivity of the fowl plague virus for mice, the preponderance of evidence indicates that the virus is pathogenic for mice. Critical reviews of the literature on the subject are presented in articles by McKenzie and Findlay[33] and by Nakamura and Imai.[34] These authors succeeded in establishing the infection in white mice and in passaging the virus serially by intracerebral inoculations of brain virus. Such virus was referred to as mouse strains or mouse variants owing apparently to an increase in virulence for mice; the virus was unaltered in its virulence for chickens.

In our experiments with the D.E.I. virus, white Swiss mice 3 to 4 weeks of age were used. The virus source was serum of an infected chicken. This chicken had been infected by intravenous inoculation of the virus in a.a. fluids from eggs of the first a.c. passage of the virus in our laboratory. Initially, 5 mice were inoculated intracranially with 10^3 embryo m.l.d.'s of the virus. Three of these were sacrificed four days later while clinically normal, and 0.03 to 0.05 cc. of the 10^{-4} dilution of the brain composite was infective for 12-day embryonating eggs. Dilutions higher than the original 1:10 emulsion of brain in buffered broth were made with the lightly centrifuged supernate. In later samples that were contaminated with bacteria, the supernate was admixed in a 1:2 dilution with a preparation of penicillin sodium containing 20,000 O. U. per cubic centimeter and allowed to remain for one hour prior to the preparation of further dilutions. One of the 2 remaining mice showed hyperexcitability and incoördination on the eighth day and was sacrificed; its brain contained an amount of virus similar to that of the first group. The remaining mouse was sacrificed fourteen days postinoculation, and virus was not demonstrated in its brain. The virus from the first group of mice and that from the one that showed symptoms on the eighth day were each used to conduct four brain to brain passages. The data for one of the passages are shown in table 4.

The brain to brain passages of the D.E.I. virus appeared to bring about a measure of adaptation to mice. This was evidenced by the lethality that occurred in the second and subsequent mouse passages, and by a decrease in the survival times of the mice to a minimum of three to four days, and by the dissemination of the virus to, and the increase of its concentration in other organs. Extracranial sites of predilection appeared to be the lungs and spleen, while in some cases smaller amounts of virus were demonstrated in the liver and kidneys and, on the few tests made, none was demonstrable in the testes. This evidence was supplemented in another experiment wherein, in four groups of 4 mice, 1 each was inoculated intracranially, intraperitoneally, subcutaneously, and intravenously with 10^3 to 10^4 embryo m.l.d.'s of the brain virus from the fifth mouse passage. For comparison, an equal number of mice was similarly inoculated with the same amount of virus contained in a portion of the same serum used to initiate the mouse passages. The mouse-passaged virus killed all of the mice within twelve days except 1 inoculated intraperitoneally which survived; the shortest survival time (three and five-tenths days average) followed intracranial injection; the longest (nine days average) followed subcutaneous injection. The original virus caused death of the 4 mice inoculated intracranially five and six days postinjection, while, with the exception of 1 mouse inoculated subcutaneously and devoured ten days later by the other mice in the group, all others survived without indications of illness. The intracranial injection of 4 mice with a mixture of brain virus of the fifth mouse passage and high titer antiserum for the D.E.I. virus was apparently responsible for the death of 2 of the mice seventeen and nineteen days postinjection; the other 2 remained normal. Only the lungs of the first mouse to die were tested for virus. None was

demonstrated. Chickens inoculated intravenously with 1, 10^3, or 10^4 embryo m.l.d.'s of the brain virus of the fifth mouse passage died of typical fowl plague.

The 4395 variant of the D.E.I. virus was apparently avirulent for mice. Of 8 mice inoculated intracranially with 10^6 embryo m.l.d.'s (intravenous route), 4 remained healthy for four weeks, or until removed from the experiment, while the other 4 were sacrificed for study five days postinoculation. Their brains were triturated and diluted 1:5 with 1 per cent peptone solution. The supernate of the composite specimen was tested for the virus by the intravenous inoculation of embryonating eggs. The presence of the virus in small amounts was shown by death and typical evidences of infection in 1 of each group of 4 embryos that received the undilated and the 1:10 dilution of the supernate.

SPECIFIC INACTIVATION OF VIRUS IN VITRO

By Serums.—Adult geese, which are relatively refractive to fowl plague virus, were found by Krauss and Doerr[35] to respond to injections of the virus by the production of antibodies which were capable of destroying *in vitro* the infectivity of the virus for chickens. The goose as well as the chicken was used by Manninger [6, 36] to obtain fowl plague antiserum, and from comparative studies of it with Newcastle disease antiserum that was prepared from geese, he concluded that the two viruses were identical. However, the serum obtained from geese was capable of inactivating only very small amounts of either the homologous or heterologous viruses.

Nakamura, *et al.*[37] employed hyperimmune serum of chickens in studies of Newcastle disease virus, of plague virus, and of several strains of virus-producing diseases that resembled them. He determined that the several unknown viruses resembled Doyle's Newcastle disease virus and were not related to Chiba strain of fowl plague virus.

In vitro neutralization was employed also by Beach[38] in distinguishing so-called pneumoencephalitis from fowl plague and in identifying the malady with Newcastle disease.

In vitro neutralization of the fowl plague virus by serum was also used by Hallauer[3, 39] as a means of testing the immunizing properties of this virus, the status of immunity in chickens, and the egg transmission of antiviral immunity.[40] The material being tested for antibodies was mixed with 100 to 1,000 minimal infective doses of chicken-propagated virus and the mixtures injected parenterally into chickens. The serum of hyperimmunized chickens, in 0.05-cc. amounts, caused inactivation of various quantities of the virus used in the tests.

The occurrence of *in vitro* inactivation of the virus was further demonstrated by Hallauer[41] and by Todd[42] in their unsuccessful attempts to render the virus relatively innocuous yet immunogenic by admixture with specific antiserum.

In our investigations, the capacity of several hundred serums to destroy the infectivity of virus was determined. With a few exceptions, in which serum from rabbits was studied, all tests were conducted with serum from chickens. The common procedure involved the admixture of equal quantities of undiluted serum and of various decimal dilutions of the virus. The mixtures were held at room temperature (25 C. to 35 C.) for periods usually more than thirty minutes but less than three hours and were then injected into the allantoic chamber of 10- to 12-day embryonated eggs. The results were computed by 50 per cent end point mortality, during the subsequent eight- to ten-day incubation period, in conjunction with changes in the embryos as described elsewhere,[7] and by comparison with comparable titration of the dilutions of the virus used in making the virus-antiserum mixtures. The results were found to be reproducible within tenfold titer limits on given specimens of serum and virus in both the fresh state and following storage in favorable environments; also with mixtures of serum and virus that had stood for periods

TABLE 3.—Disinfective Action of Certain Chemical Agents on the Fowl Plague Virus at 27 to 30 C

Disinfectant		Results*		
Name	Concentration**	Virus active, minutes	Virus inactive, minutes	pH of virus-disinfectant mixture
Cresol	1:1,000	Not done	8	7.5
Phenolor	1:2,000	10	20	7.3
Clorox	1:1,000	8	15	7.1
Mercuric chloride	1:10,000	10	15	6.8
Potassium permanganate	1:1,000***	5	10	7.5
Lye	1:120	5	10	11.6

*The results were obtained by inoculation of embryonating eggs at from five- to ten-minute intervals.
**The concentrations are those that obtained after admixture of equal parts of a solution of the disinfectant in distilled water with a 1:10 dilution of the virus material (a.a. fluid in distilled water).
***In this case, a 1:5 dilution of the virus material was used.

up to twenty-four hours and at temperatures several degrees above or below the room temperature.

Comparison of Titrations in Eggs and Chickens.—Since all of the reports, previously reviewed, that have been made on the titration of neutralizing antibodies for fowl plague virus had utilized the chicken as the test organism, a comparison was made of the results obtainable with embryonating eggs and with chickens. The embryonating eggs and the chickens were inoculated by the a.c. and the intramuscular routes, respectively. It is evident from the protocol in table 5 that greater titration values for antibodies were demonstrable in chickens than in embryos. The data indicate also that the immunogenic property of the mixture of virus and antiserum in chickens is a better criterion than the lethal effect for computing end points for living or infective virus.

The practicability of the use of embryonating eggs and the reproducibility of results obtainable with them made their use the test organism of choice for the majority of our studies.

Cross Neutralization of Suspected Fowl Plague Virus Strains and the D.E.I. Virus.—The occurrence of disease processes in embryonating eggs that were atypical for infection due to fowl plague virus, as previously recorded, suggested the possibility of variation in the D.E.I. strain of virus. Following the isolation of an agent from each of 5 embryos in which this atypical condition occurred and the observation of the transmissibility of the agents to other groups of embryos, neutralization tests were conducted with serum of fowl plague-susceptible and of fowl plague-immune chickens. Only serum from the immune chickens had an effect on the virus; antiserum of high titer (0.05 cc. neutralized 10^5 to 10^7 m.l.d.'s of the D.E.I. virus) abolished the embryo infectivity of undiluted a.a. fluid from eggs lethally infected with the agents (10^4 to 10^5 embryo m.l.d.'s/0.05 cc.). With two of the variant viruses, namely 4395 and 19493, numerous subsequent tests with chicken serum verified this relationship.

Chickens and rabbits inoculated with the 4395 and the 19493 variants developed neutralizing antibodies in their serum for the parent strain of D.E.I. virus. On a virus unit basis, the immunogenicity of formalin-inactivated variant and parent viruses was essentially the same in chickens, while in rabbits, the immunity response was greater for the parent than for the variant virus. In chickens, the response to the living variant was seldom equaled by that of the parent virus except when it was inoculated subsequent to infection of the inactivated virus.[8]

Serum of normal rabbits, in 0.05-cc. amounts, was found to inactivate as much as 10^4 embryo m.l.d.'s of the variant virus used in the study with rabbits; these same serums had no demonstrable effect on the parent virus.

Serum Diagnostic Titers.—The characterizations of fowl plague, especially the ease of demonstrating the causative virus, have apparently precluded, to a large extent, the need for the establishment of means for serologic diagnosis.

The results of our study of serums of many chickens during the investigations on this disease revealed a repetitious inability to alter the infectivity of the D.E.I. virus and, in fewer tests, that of the variant viruses isolated from it. Serums from chickens of various ages obtained from flocks in Massachusetts and New Hampshire or flocks that were hatched and maintained in isolation quarters in the laboratory, serums from chick-

TABLE 4.—Data on Five Brain-to-Brain Passages of Fowl Plague Virus in Mice

Passage No.	Inoculums: Source of virus	Inoculums: Embryo m.l.d.	Time and manner of death of mice*	Embryo-infectivity titers of mouse tissues**: Brain	Lung	Liver and/or spleen	Kidney	Testes
1	Chicken serum	10^3	K4, K4, K4 SK8 K14	10^{-4} 10^{-4} —	— — —	10^{-1} —	— —	—
2	K4 K4 K4	10^2	K5, K5, K5 D6, D7 D8	10^{-6} 10^{-7}				
3	K5 K5 K5	10^4	D4, D4, D5 D5, D8, D9	10^{-5}	1:20	1:20, —, ND	1:20 1:20, ND	
4	D4 D4 D5	10^3	D4, D4, D5 D6, D7, D8	10^{-5}	10^{-4}	1:20 10^{-3}	1:20	
5	D4 D4 D5	10^3	D4, D4, D4 D4, D4, D4	10^{-9}	10^{-4}	1:20 10^{-4}	1:20	—

*K = Mice killed while apparently healthy; D = Death; SK = Nervous symptoms present when killed; numerals indicate days postinoculation when the mice showed symptoms, died, or were sacrificed.
**— = Virus not demonstrated by test; blank space = test for virus not made; ND = test not made for one mouse in a group; and where a single titer is recorded for any tissue, the result applies to a test of a composite of that tissue in the event more than one mouse was involved.

ens exposed by simulated natural means to fowl plague and which escaped death, and serums from chickens immunized against Newcastle virus gave no interference with the infectivity of the fowl plague virus when compared in the usual manner with the titrable end points of infectivity of the virus. Accordingly, the usefulness of the test would appear to be confined largely to the occurrence of less severe forms of the disease, as produced by agents that are suggested by the variant strains described; or to the occurrence of the classical disease in vaccinated flocks, since in both cases unusually high titers of neutralizing antibodies would be expected to occur.[8]

Stability and Preservation of Immune Serum.—Tests were conducted to determine the stability of infectivity-neutralizing antibodies for the fowl plague virus under various conditions, but particular attention was given to time, temperature, and variables of physical state of serums involved in the performance of all investigations on fowl plague. Portions of each of three serums with initial neutralization titers of 10^6 m.l.d.'s of virus/0.05 cc. were stored, in flame-sealed small tubes, in the crude form at 6 C. and –70 C. and in the lyophilized form at –70 C. Neutralization tests conducted eleven, twenty-five, and thirty-nine days later, using portions of the same virus preparation in all tests, revealed tenfold lower titers in one half of the tests; the diminution was not correlated with any of the three major variables. In further tests of the serums stored for as long as nine months, titers were obtained similar to the initial ones. A portion of one of the serums stored in the crude form for six and one-half months at 37 C. maintained its antibody titer. Another serum maintained its full potency when stored in the lyophilized state at –70 C. for twenty-three months.

The effect was determined of phenol and of merthiolate on the infectivity-neutralizing antibodies. Concentrations of these agents were employed that were considered of possible use in preventing decomposition of serum by microörganisms and that were determined to have no deleterious effect on chicken embryos. Four levels of phenol, including a maximum concentration of 1 per cent, and 0.01 and 0.02 per cent levels of merthiolate were incorporated in bacteriologically sterile serum from normal and from plague-immune chickens. Portions of the serums were diluted to a comparable degree with a physiologic solution of sodium chloride. Each 0.05 cc. of the specimen containing the immune serum neutralized 10^7 m.l.d.'s of the virus. The serums were stored at 6 C. in rubber-capped tubes. Preliminary and succeeding tests of the normal serum showed that the preservative alone had no effect on the titer of the test virus, and tests of the immune serum at intervals during six months of storage revealed that neither the phenol nor the merthiolate had any adverse effect on the antibody titer.

Egg Yolk Antibody Titers.—Eggs laid by hens that had an acquired resistance to fowl plague were observed by Hallauer[40] to contain neutralizing antibodies in the yolk for the fowl plague virus. Other literature on egg transmission of antibodies has been reviewed in a previous paper, and the occurrence of Newcastle virus antibodies in the yolk has also been established.[43]

The results of our studies largely confirmed those of Hallauer,[40] being different only in matters of detail related to higher antibody titers and to our use of embryonating eggs rather than chickens as the test organism. The albumen and the yolks of the freshly laid eggs of 6 hens immune to fowl plague and of 2 susceptible hens were each diluted 1:5 with a physiologic solution of sodium chloride and tested for infectivity-neutralizing antibodies. Five-hundredth's cubic centimeter of the yolks of the 6 immune hens inactivated 10^4 to 10^6 m.l.d.'s of virus, while the albumen of these eggs was without effect on the virus. The antibody titers of the yolks corresponded closely to those of the serums of the respective hens. Neither albumen nor yolk of the susceptible hens' eggs altered the infectivity of the virus. The usefulness of this information for the purposes of diagnosis of this disease would be limited by the same considerations as described in a previous section for serums.

Specific Neutralization of Virus Hemagglutinin.—The abolition of the agglutinative action of the fowl plague virus for chicken erythrocytes by specific antiserum was reported by Lush.[34] This author observed also the inability of antiserum for the Newcastle virus to affect the fowl plague hemagglutinin and, in turn, the specificity of that antiserum for the Newcastle virus.

The observations of Lush were confirmed early in the course of our studies, using fowl plague and Newcastle virus antiserums prepared in both rabbits and chickens. Immune serums obtained for two of the plague-variant viruses likewise inhibited the hemagglutinin of the D.E.I., or parent strain.

Exact quantitative relationships were not established between the infectivity-neutralizing and the hemagglutination-inhibition titers of the serums. However, the serums commonly used neutralized in excess of 10^4 embryo m.l.d.'s of virus/0.05 cc., and the virus preparations of embryo origin caused agglutination in 1:160 or greater dilutions. In a study of the serum of hyperimmunized chickens (0.05 cc. of serum neutralized 10^6 m.l.d.'s of virus), a 1:40 dilution of the serum caused complete inhibition of 1:10 and greater dilutions of a virus material with an agglutination titer of 1:320. The use of further dou-

bling dilutions of the serum to 1:1,280 caused a diminution in the inhibition to its end point in a linear manner according to various serum inhibition phenomena.

CROSS IMMUNITY TESTS

The widest application of procedures of cross immunization involving fowl plague has occurred since the time of the first recognition of another virus disease, namely Newcastle disease, that bore some resemblance to it. Thus, the dissimilarity of fowl plague and the malady that came to be known as Newcastle disease was established by Doyle.[4] In turn, subsequent outbreaks of disease in England, the Dutch East Indies, the Philippines, Australia, India, Korea, and Japan were found to be unlike fowl plague and were identified with the Newcastle disease virus that was isolated originally.[41] However, an atypical fowl plague in Egypt that was described by Lagrange[3] and was considered as possibly related to Newcastle disease was shown by Purchase [43] to be related immunologically to fowl plague.

Tests with Newcastle-Immune Chickens and Strains of Plague-Variant Virus. — Our studies, as previously recorded,[24] were undertaken to verify the identity and purity of D.E.I. strain of the fowl plague virus, which served for the inauguration of our work. Chickens were immunized against the respective viruses with vaccines prepared by formalin-inactivation of the egg-propagated viruses as described in other papers [5, 46] or otherwise as specifically indicated. Challenge exposure of the chickens to 10^3 or 10^4 m.l.d.'s of the viruses intravenously resulted in death of those immune (8 chickens) to Newcastle disease that received the fowl plague virus and of those (21 chickens) immune to fowl plague that received the Newcastle virus. The ones (12 chickens) immune to Newcastle virus and challenged with the homologous virus survived. Ten of the plague-immune chickens had been vaccinated and had, in addition, withstood an exposure to the same virus that was uniformly lethal to the controls. The nature of the disease that occurred in the immune chickens exposed to the heterologous viruses was comparable to that produced in each group of 10 stock chickens by the respective viruses.

The results of initial studies[19] on the immunogenicity of the living variant viruses derived from the D.E.I. strain typified the strong evidence for their antigenic relationship to the parent virus. Of 18 3-month-old chickens that received from 2 to 20 x 10^5 embryo m.l.d.'s of the 4395 variant, 14 survived and were exposed by the same route to 100 m.l.d.'s of the parent virus twenty-one days later. All 14 survived this challenge, while 2 controls died at twenty-two and twenty-eight hours postexposure. Likewise, of 21 39-day-old chickens injected intravenously with from 10 to 10^7 embryo m.l.d.'s (by tenfold increments) of the 19493 variant, all survived and were exposed one hundred and five days later by the same route to 10^4 m.l.d.'s of the D.E.I. virus. Sixteen of these chickens survived the challenge without apparent illness, while the other 5 immunized birds died of chronic illness four to eleven days later. The 3 controls died in twenty-four hours. The low virulence of the variants precluded an assessment of their effect on chickens immunized against the D.E.I. virus except as indicated by a marked augmentation in antibody response.[6]

An attempt to compare, on a unit basis, the antigenicity of the formalin-inactivated 4395 variant virus with that of the formalinized parent virus was confined to a single intramuscular injection of groups of 3 or 4 chickens each with graded amounts of the two inactivated viruses. When their resistance was challenged ninety-eight days later by 10 or 10^3 m.l.d.'s of the D.E.I. virus, only 2 of the chickens vaccinated with the variant survived; these had received, along with 1 other chicken, the maximum amount of the variant that was injected, or 10 cc. of the virus preparation which contained 2 x 10^6 units of the virus (calculated from the initial infectivity titer). A comparable amount of the formalin-killed parent virus, 2 x 10^6 units (0.1 cc.), failed to protect any of 4 chickens against the challenge exposure. One half of the chickens that received 2 x 10^5 (1 cc.) and all 4

TABLE 5.—Comparison of 12-Day Embryonating Eggs and 39-Day-Old Chickens for Evaluating Inactivation of Fowl Plague Virus by Specific Serum

Inoculum		Results		
		In eggs	In chickens	
Embryo m.l.d. of virus (/0.05 cc.)	Serum	S/E Ratio*	S/E Ratio	S/E Ratio to subsequent intravenous injection of 100 m.l.d. of virus
10^8	0.05 cc.	2/5	5/5	5/5
10^7		3/5	5/5	3/5
10^6		5/5	4/5	1/4
10^7	None	0/4	0/1	
1		1/4	0/1	
0.1		4/4	1/1	0/1

*S/E Ratio is the number of survivors(S)/ the number of test organisms exposed (E).

chickens that received 2 x 10^{10} (10 cc.) of the killed D.E.I. material survived the challenge exposure. All 4 controls died of typical fowl plague. While differences in antigenicity on a unit basis may have been obscured by the possible adjuvant effect of the quantities of egg material that were injected, it appeared that the survival of the 2 variant-vaccinated chickens was evidence of immunologic relationship to the parent virus.

DISCUSSION

For many years, the diagnosis of fowl plague on a virologic basis necessitated proof only of the presence of a virus in an epizoötic disease of short clinical duration. However, this criterion became inadequate with the appearance and/or recognition of atypical forms of this disease and of Newcastle disease, all of which were observed to manifest likenesses to one another. While many studies had been conducted and much useful information had been obtained on means for the virologic and other differentiation of the causal viruses and the diseases they produced, it was apparent that extended considerations were desirable in order to provide accurate and expeditious means of diagnosis. The foregoing experimental study of fowl plague was conducted concurrently with a study of Newcastle disease, as previously reported. The studies reported on fowl plague were primarily designed to obtain information bearing on immunization against this disease, as reported in another paper.[8]

The embryonating chicken egg was used almost exclusively as the test organism for the detection of the virus and for the maintenance of supplies of the virus materials used in the studies. The observation of others on the easy adaptability of the virus to the chicken embryo was verified.

The long recognized pantropic nature of the disease in chickens was readily ascertained. Additional evidence in this regard was obtained by tests of materials collected at intervals antemortem. It was shown that virus was demonstrable fifteen to eighteen hours postinoculation in association with the blood cells, and that virus made its appearance within six to nine hours later, or approximately ten hours prior to death, in the nasopharyngeal mucus, plasma, and feces. The importance of proper storage or selection of materials to be used for diagnostic tests was emphasized particularly in regard to liver tissue, wherein, either in intact liver or in diluted triturates thereof, the virus disappeared in about one week under storage at 37 C. and was reduced (most markedly in intact liver) in titer at 6 C., but retained its original titer at –70 C.

The virus could be recovered from the tissues, secreta, and excreta of chickens affected with the acute form of the disease, but not from chickens affected with the chronic forms produced by exposure of immunized chickens to the virulent D.E.I. virus. It would, thus, appear that, if present, the virus in the tissues of the chronically affected chickens studied was so effectively masked as not to be detectable by the procedures and more effectively masked than in the studies reported by Hallauer[9] in which the coexistence of antibodies and virus was demonstrated during the delayed period of clinical disease produced by active-passive immunization from which the chickens recovered.

The isolation of variant strains of virus from the D.E.I. strain and the definition of certain of their features would appear to signify the ability of the fowl plague virus to undergo alterations. All of the variants were isolated from embryos inoculated with end point infectivity dilutions of materials containing the D.E.I. strain, and no correlation was established between the nature of the source materials, their history, or other factors and the likelihood of yielding variant strains.

The possibility that the variants might be of more common occurrence than was indicated by deaths of embryos was not verifiable by blind passage in embryos with spleens of embryos that survived inoculation with end point infectivity dilutions of the D.E.I. virus materials or with spleens of embryos that were injected intraallantoically with 1 to 10 intravenous embryo m.l.d.'s of the variants. Neither could this be demonstrated by challenge of the immunity of 3-week-old chickens that had hatched from eggs inoculated with sublethal amounts of the variant.

Attention was given to such features of the D.E.I. strain, and to the variants to a

lesser extent, as filterability, tolerance to penicillin, tenacity to heat and various conditions of storage, sensitivity to ultraviolet light and to various chemical agents, pH stability, hemagglutinative activity, and mouse infectivity. The tolerance of the virus for penicillin permitted the use of this agent for the suppression of contaminants in various inoculums but particularly for studies on the isolation of the virus from feces and nasopharyngeal mucus of chickens. The pattern of pH stability of the D.E.I. virus, its hemagglutinative activity, and infectivity for mice would permit its differentiation and even physical separation from the virus of Newcastle disease and undoubtedly, also, from other avian viruses. In connection with studies on the hemagglutinin, a procedure is described for rendering erythrocytes insensitive *in vivo* to hemagglutinins of homologous virus. In comparative studies with the virus of Newcastle disease, the hemagglutinin of this virus was inactive for plague-infected cells, whereas the Newcastle-infected cells were agglutinated by the plague virus.

The principles for immunologic recognition of the plague virus were reviewed and applied to ascertain the purity and identity of the D.E.I. virus and for standardization or interpretation of procedures used in studies on immunization. The procedures were applied, also, to establish the identity of the variant viruses that were isolated from the parent virus.

SUMMARY

A report is made of an investigation of methods of isolation and identification of a Dutch East Indies strain of the fowl plague virus and of five variant viruses that were isolated from it.

Embryonating chicken eggs were used extensively for the isolation and continued propagation of the viruses. The known pantropic nature of the classical disease was substantiated along with the widespread distribution of the virus postmortem in chicken embryos and chickens. Specific information was obtained on the appearance of the virus in the secreta, excreta, and blood before death. Consideration of the identification of the viruses was given largely to the stability of the viruses in the presence of penicillin, of various chemical agents, and of various physical factors, to the characteristics of the hemagglutinin, to the infectivity of the viruses for mice, and to various immunologic aspects.

The following features were observed for the D.E.I. virus: (1) tolerance to high concentrations of penicillin which were effective and useful for the suppression of organisms that otherwise interfered with the isolation of the virus from contaminated materials such as chicken feces; (2) tolerance to concentrations of phenol and merthiolate effective in maintaining asepsis in chicken serums intended for serologic use and which in neutralization tests did not affect adversely the antiviral substances; (3) sensitivity to heat and concentrations of certain disinfectants within ranges of their practical usage; (4) marked variations in tolerance of the infectivity, but not in the uniformly high resistance of the hemagglutinin, to intensities of ultraviolet light associated with different wave lengths; (5) a much narrower range of stability in an acidic as compared to an alkaline environment; (6) a hemagglutinin active for a wide range of species' erythrocytes and for erythrocytes infected with the virus of Newcastle disease but not for those infected with the homologous virus, and sensitive to inactivation by specific antiserum; (7) a native infectivity for mice when inoculated by the intracranial route, but with a capacity for further adaptation to mice without alteration in virulence for chickens; (8) a capacity to vary in a manner that permitted the isolation of relatively avirulent but immunogenic variant viruses; (9) inactivation *in vitro* by specific antiserum; and (10) inability, as a rule, to produce lethal infection in chickens immunized with inactivated, egg-propagated, homologous virus or with the inactivated or living variant viruses that were isolated from it.

The features that were determined for the variant viruses which were in contrast to those of the D.E.I. virus were as follows: (1) less virulent for embryonating chicken